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ABSTRACT

Because of the daily impact of biotechnology, it is important that students have some knowledge and experience with biotechnology in order to enable them to deal with the issues that arise as a result of its implementation. The purpose of this workbook is to assist in the efforts to expose students to the concepts of biotechnology through hands-on project activities. The projects in this book represent ideas that may be used as a starting point for a student project. These projects may be modified and adapted to suit each situation. In some cases, the student will have to work at a local university or research lab. Suggested student research projects in the following areas are presented: (1) Biotechnology in Food Production, (2) Biopolymer Synthesis, (3) Plant Biotechnology, (4) Enzyme Expression and Production, (5) Altering Genetic Characteristics, (6) Applications of DNA Analysis, (7) Protein Analysis, (8) Industrial and Environmental Biotechnology, and (9) Cellular Physiology. (PR)

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The Ohio Science Workbook:

BIOTECHNOLOGY

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Compiled by
SPENCER E. REAMES
for The Ohio Academy of Science

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The Ohio Science Workbook:
BIOTECHNOLOGY

The purpose of this workbook is
to assist in the effort to expose students
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Spencer E. Reames

for

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The National Science Foundation, The Ohio Academy of Science and the authors do not assume liability for research risks (health and safety) for anyone using projects or other information or ideas from this publication.

Projects should be conducted under proper supervision using prudent laboratory health and safety precautions.

FOREWORD AND ACKNOWLEDGEMENTS

This book is an experiment based on the assumption that students who wish to pursue research should have access to the latest ideas from the world of governmental, industrial and academic research. Working with researchers in biotechnology centers in Ohio, many other states and around the world, Mr. Spencer Reames has compiled ideas for projects from the simple to the sophisticated. This book is a **tool** to provide access for students to research ideas and to enable students to seek additional information and understanding about biotechnology.

In developing this and similar workbooks on polymer science and manufacturing science we have encountered some skepticism from researchers as to the ability of precollege students to perform the work proposed in this workbook. I'm afraid that the research community has been too easily confused by media headlines of the decline of science education in America. While there is little doubt about the deplorable conditions in many schools, we already know that many Ohio students are already conducting world class research. Clearly, significant projects can be developed when bright and open minded students work with enthusiastic teachers and researchers. I fully expect to see some of these ideas from this workbook showing up in projects at our own State Science Day, at The International Science and Engineering Fair and in the Westinghouse Science Talent Search.

Perspective, persistence and patience are the hallmark words in the development of this book. Our perspective on biotechnology is worldwide and broad. It encompasses such areas as food production, biopolymer synthesis, plant biotechnology, enzyme expression and production, altering genetic characteristics, applications of DNA analysis, techniques, protein analysis, industrial and environmental biotechnology, and cellular physiology.

Mr. Reames, who compiled these projects and did most of the editing, has been persistent in making sure we have included a broad range of projects. He has been persistent in sticking with the development of this book. We appreciate the patience of the authors and others who anticipate using the book. As with many projects, this one has taken longer than expected.

Acknowledgements

I thank the many authors for their submissions of project ideas which are the heart of this book. I hope they will be pleased with the results of their work.

I also thank Dr. John Finer of The Ohio Agricultural Research and Development Center for writing the summary of NIH guidelines.

Mr. Bruce A. Leach, Librarian at The Ohio State University Biological Sciences Library, helped verify the accuracy and completeness of bibliographic information in the annotated references section.

The excellent proof reading services of Ms. Sue Wenig helped make the first draft of this workbook faithfully reflect the original submissions of the authors. Ms. Kim Perry, who handled all of the word processing, also provided essential interpretation of sometimes difficult to read handwriting and notes of those involved in editing, and to improve upon the formatting of the document.

I also appreciate the continuing interest in this project from the directors and staff of the Ohio's Edison Program biotechnology centers and from Dr. Pappachan E. Kolattukudy, director, The Ohio State Biotechnology Center.

To assure adequate and accurate scientific content, this book was reviewed by more than 60 people in industry, government and academia from over 20 states and countries. Every

project had at least three reviewers; some projects were reviewed by 6-8 reviewers. Affiliations of those reviewers, listed here, are supplied for acknowledgement and recognition purposes only. Endorsement of this book by any of these reviewers or by their employers is not implied. Listed alphabetically by last name, the reviewers included:

Dr. Rod Anderson, Ohio Northern University, Ada OH; Mr. Steven M. Anderson, Roche Biomedical Laboratories, Research Triangle Park, NC; Mr. Bill Bahr, Southwestern High School, Patriot OH; Mr. Jonathan Bealer, Hereford AZ; Mr. Richard Benz, Wickliffe High School, Wickliffe, OH; Mr. William R. Bingle, Perry Middle School, Massillon OH; Ms. Pat Brock, St. Louis Mathematics and Science Education Center, St. Louis MO; Mrs. Kathleen J. Bunn, Cuyahoga Falls OH; Dr. Agustin Lopez-Munguia Canales, CIIGB-UNAM, MEXICO; Mr. Robert E. Cannon, University of North Carolina, Greensboro NC; Ms. Elizabeth Carvellas, Colchester VT; Mr. Edward L. Corley, Eaton High School, Enon OH; Mr. Michael A. Cotta, USDA-Agricultural Research Service, Peroia IL; Dr. Keith R. Davis, OSU-Biotechnology Center, Columbus OH; Mr. Peter DeDecker, Hastings High School, Hastings MI; Dr. Joyce Durnford, Battelle Memorial Institute, Columbus OH; Mr. David Ely, Champlaign Valley Union High School, South Burlington VT; Dr. R. C. Evans, Rutgers University, Camden NJ; Dr. Park K. Flick, United States Biochemical Corporation, Cleveland OH; Prof. E. W. Frampton, Northern Illinois University, Dekalb IL; Dr. M. O. Garraway, OSU - Dept. of Plant Pathology, Columbus OH; Dr. Mark B. Gorman, Baldwin-Wallace College, Berea OH; Dr. Mary M. Grula, Oklahoma State University, Stillwater OK; Dr. N. F. Haard, University of California Davis Food Science Dept., Davis CA; Dr. Michael S. Herschler, Otterbein College, Westerville OH; Dr. Dallas G. Hoover, University of Delaware, Newark DE; Dr. David P. Houchens, Neoprobe Corporation, Columbus OH; Ms. Judy Johnson, University of Nebraska, Lincoln NE; Dr. Karl Joplin, OSU-Dept. of Entomology, Columbus OH; Dr. Pablo S. Jourdan, OSU College of Agriculture, Columbus OH; Dr. Cinda Herndon-King, Edison Biotechnology Center, Cleveland OH; Ms. Judy Lachvayder, Parma High School, Parma OH; Dr. Christine Lang-Hinrichs, Federal Republic of Germany; Dr. George H. Liang, Kansas State University, Manhattan KS; Mrs. Joanne Zinser Mann, Gahanna Public Schools, Gahanna, OH; Mr. John Markwell, University of Nebraska, Lincoln NE; Mr. Gordon Mendenhall, Ball State University, Indianapolis IN; Ms. Pat Ortli, Benjamin Logan Local Schools, Bellefontaine OH; Dr. Adrian J. Parr, Norfolk UK; Dr. James R. Pease, Boehringer Mannheim, Indianapolis IN; Dr. Kent Peters, OSU Biotechnology Center, Columbus OH; Dr. N. K. Ranaswany, OSU Biotechnology Center, Columbus OH; Dr. Keith Redenbaugh, Calgene, Davis CA; Dr. Jerald Silverman, OSU - Lab. Animal Center, Columbus OH; Ms. Ruth A. Simione, American Type Culture Collection, Rockville MD; Dr. Diane Spindler, Solar Energy Research Institute, Golden CO; Mr. Jeff Steele, Mapleton High School, Ashland OH; Mr. Fred Stutzenberger, Clemson University, Clemson SC; Mr. Richard Thieret, Trimble High School, Athens OH; Ms. Mary Tierny, OSU Biotechnology Center, Columbus OH; Mr. Antonio Garcia Trejo, Chandler AZ; Dr. K. K. Ussuf, OSU Biotechnology Center, Columbus OH; Dr. Peter A. Vandenbergh, Microlife Technics, Sarasota FL; Dr. Carolyn N. Vann, Ball State University, Muncie IN; Dr. V. Venugopal, Bhabha Atomic Research Centre, Bombay INDIA; Dr. Cheng-i Wei, University of Florida, Gainesville FL; Dr. Michael E. White, OSU - Dept. of Animal Science, Columbus OH; Dr. Daniel R. Zeigler, Bacillus Genetic Stock Center, Columbus OH; and Tom Zupancic, Battelle Memorial Institute, Columbus OH;

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Lynn Edward Elfner
Chief Executive Officer
The Ohio Academy of Science
March 1993

PREFACE

Biotechnology is having an ever expanding impact on each of us. This impact has not gone unnoticed by those who study changes in society. John Naisbitt and Patricia Aburdene (1990) noted in their book *Megatrends 2000* that biotechnology is leading us into an "Age of Biology" and that the new biology which has flourished as a result of the technological advances associated with biotechnology, will be one of the ten most important factors influencing our society and world in the coming decade. These advances and changes are global.

Because of the daily impact of biotechnology it is important that students have some knowledge and experience with biotechnology and the new biology in order to enable them to deal with the issues that arise as a result of their implementation. Cold Spring Harbor Laboratory has been a leader in getting DNA science into the classrooms of this country. They have been joined by a number of universities and individuals across the country. **These efforts are having a tremendous impact on setting a new agenda for secondary school life sciences curricula.**

The work of Cold Spring Harbor Laboratory and others, such as The Georgetown University group, and The North Carolina Biotechnology Center has resulted in increasing the level of knowledge, interest, and enthusiasm about DNA and related biotechnologies. Because of the editorial staff's broad based approach to biotechnology, a number of other types of projects such as biopolymer production and fermentation will be found in this workbook in addition to DNA related projects. This broad approach reflects the types of activities that occur in academic research and industrial research by those who consider themselves biotechnologists.

A NOTE TO TEACHERS

The projects in this book represent ideas that may be used as a starting point for a student project. These projects may be modified and adapted to suit your situation. In some cases, the student will have to work at a local university or research lab in order to conduct the research.

It is assumed that you will work with students as they pick projects from the book, explaining to them what the project is about and how one might go about getting started. If students are left to start on their own without input and encouragement, the project often never gets off the ground. The teacher will often find it necessary to help line up equipment or make arrangements for the student to work with an outside mentor. This workbook was never meant to be a stand-alone resource. It was meant to stimulate thought and ideas. In some cases the projects are asking the students to develop an idea or technique from scratch. Obviously projects of this type are of a much more sophisticated level when compared to other projects

The purpose of this book is to assist in the effort to expose students to the concepts of biotechnology through hands-on project activities. This workbook differs from other publications that have been offered to teachers and students because it takes a broad view of biotechnology and it focuses on student involvement with independent research projects. It is the sincere hope of The Ohio Academy of Science and editorial staff that this book will stimulate student research and as a result develop student interest in biotechnology and biology as well as aid the student in experiencing the excitement that one has when engaging in scientific research in the pursuit of answers to challenging questions.

During the production of this book, a broad definition of biotechnology was used and a global perspective on biotechnology was taken. This is evidenced not only by the types of projects which appear in the workbook, but also in the number of states and countries from

which these projects were submitted. In no way should one think that the projects in this book represent the whole of biotechnology. These projects represent a flavor of how biotechnology is used to address a multitude of questions,and these applications will only expand as our knowledge increases.

As you look through the workbook, you will find that the projects in related areas are grouped together. In general as you progress within each section of related projects the level of sophistication and difficulty increases. In some cases, projects are placed at the more sophisticated level due to the equipment requirements of the project. In these cases we encourage the student to work with a scientist at a research lab or university. In some cases, it may be possible to modify the project so that the student may conduct the project more easily.

Before beginning a project, the student should become familiar with the techniques required, safety issues related to the project, and technical information. By planning ahead, developing the protocol, collecting the necessary materials before starting, and allowing adequate time, the student will have greater success and a more meaningful experience.

Spencer E. Reames
March 1993

Safety Concerns and Issues

When working in a laboratory situation, there is always a potential for accidents or injury. For this reason, it is important that close attention be paid to the utilization of proper techniques for handling microbes and chemicals. If the student or teacher is unfamiliar with a microbe, chemical or technique, they should familiarize themselves with these before beginning the lab work. If this is done and care is taken along with thinking before acting, the potential for accidents and injury are held to a minimum.

Many of the projects in this workbook utilize microbes. When working with microbes and cell cultures, it is absolutely crucial that good aseptic technique be utilized. Before beginning the lab work, students should become familiar with good aseptic technique. This would include disinfecting the surface area before and after use, WASHING HANDS BEFORE AND AFTER LAB WORK, as well as these following practices:

1. Access to the laboratory is limited or restricted at the discretion of the instructor when experiments are in progress.

2. Work surfaces are decontaminated after any spill of viable material.

3. Mechanical pipetting devices are used (**mouth pipetting is prohibited**).

4. Eating, drinking, smoking, and applying cosmetics are not permitted in the work area.

5. All procedures are performed carefully to minimize the creation of aerosols.

6. It is recommended that laboratory coats BE WORN to prevent contamination or soiling of street clothes.

7. All cultures should be sterilized after use.

Students should be given instruction on how to read a Material Safety Data Sheet (MSDS) for chemicals. These should be available for students to look at before they work with a chemical with which they are not familiar. Teachers should ensure that the student knows how to handle chemicals safely. A procedure should be in place in each lab (for the student to follow) in the event of a spill or accident. This may be as simple as to immediately notify the teacher. All students be informed as to how to use safety showers, eyewashes etc.

When working with irritating or potentially toxic chemicals, lab coats, safety goggles, gloves, and in some cases masks should be worn to protect the user. Before working with a chemical, the students should be familiarized with the potential dangers (if any) of the chemical. Organics should be worked with in a fume hood to prevent exposure and build-up of vapors.

If ethidium bromide is used to stain DNA, the staining should be done by the teacher in an area that is not accessible by students. From a safety point of view it is advisable to start with ethidium bromide which has already been dissolved in water (this prevents exposure to the toxic dust).

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SELECTING A PROJECT

As you look through the projects in this book, it is important to keep in mind that the projects are outlines. They can be modified and altered to meet the conditions under which you work, your interests, and to address questions which may develop as the project progresses. Scientists often modify their research for the same reasons.

The selection of a project is an important process and it should not be trivialized. You should select a project or topic that is of interest to you and not because a project is of interest to your friend. You are the one that will be working on it and unless your interest is real, you probably will not do your best work.

How to Find Information on Biotechnology

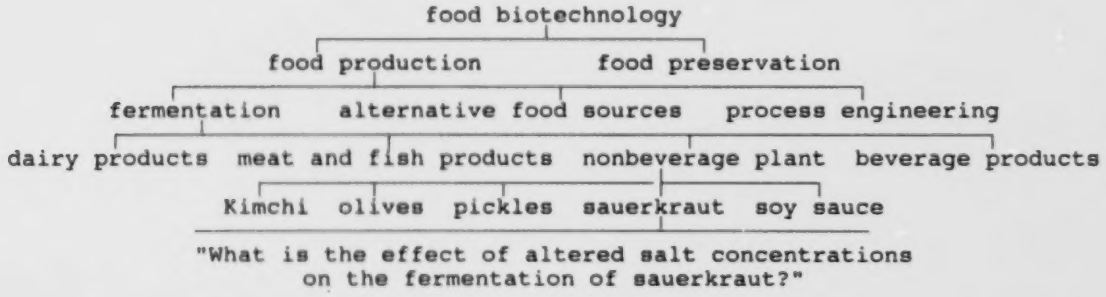
Although sorting through the maze of publications and information sources on biotechnology can prove to be difficult work, you may circumvent many of the difficulties involved in finding information if you narrow your topic. One of the most serious errors of first time researchers is trying to do too much. Your information search and your project will go much better if you keep this in mind.

For example, if you have chosen food biotechnology as a general area of research, and if you are trying to formulate a question or problem, you need to understand that information can be broken down into smaller units. Soon you'll reach an area that is limited enough to allow you to proceed until you have exhausted the available sources of information.

Biotechnology is a very broad topic and it can be divided into many areas such as environmental biotechnology, genetic engineering, food biotechnology, as well as other subdivisions. Each of these areas can be further divided into topic areas. For instance, if you are interested in food biotechnology, you might divide the topic into two broad topics of food production and food preservation. Food production might be divided into the topics of fermentation, alternate food sources, and bioprocess engineering. If you are particularly interested in fermentation in food production, you could divide fermented foods into dairy products, meat products, nonbeverage plant products, beverage products, and breads. Projects on fermented nonbeverage plant products, for example, could deal with Kimchi, olives, pickles, sauerkraut or soy sauce. The project could focus on one of these products. You may focus your research on:

"What is the effect of altered salt concentrations on the fermentation of sauerkraut?"

The following diagram illustrates the concept of narrowing the topic.



Once the topic has been narrowed, an hypothesis may be formed (see developing hypothesis and objectives), and an experiment is designed to test the hypothesis. In the case of the sauerkraut fermentation example, A 0% salt concentration in cabbage would have to be included to serve as the experimental.

If you have selected a project from the workbook, in most cases the topic of the project has already been narrowed for you. It is possible in some projects, such as the sauerkraut fermentation project to narrow the topic even more. You could determine the effect of salt concentrations on a single microbe associated with sauerkraut production.

When doing a project in the workbook, it is important that you not only use the references at the end of the project, but that you expand your search for relevant information to other publications and sources of information. During your search you will discover new terms which relate to biotechnology. Be sure that you find the meanings of these terms so that you develop a clear concept of what you have read. There is a glossary at the back of this book to help you with this process. This ensures that you have complete and up to date information about your project.

Using the Library

Let's look at how you might use your local library to find information. Although advanced research requires access to university and research libraries, you'll find that your local library will give you access to a world of information if you'll only ask.

Card Catalog

The first stop in the library will probably be the card catalog. Here you'll find, arranged by author, title, and subject, a complete listing on cards of the books available in the library. Some libraries now have computer terminals with "on-line" catalogues which may be searched rapidly for publications related to your topic. These "on-line" catalogues often are useful to find publications which, although not in the library you are visiting, may be borrowed from other libraries through inter-library loan. The card catalogue will list circulating books, which are usually stored on long rows of shelves or stacks. You can also use the card catalogue to find non-circulating reference books.

Reference Books

Reference books include science encyclopedias which can be used as a starting point in your research to get an overview of the subject. A top quality research project, however, must use many other sources of information since encyclopedias are general and seldom are useful beyond the early stages of your work.

Handbooks of standards, formulas and data will be helpful in determining whether your results are similar to those obtained over the years by other researchers. Handbooks also provide information on mathematical conversions and basic chemical and physical science data such as constants and properties of materials.

One type of reference book that is often required for biotechnology related projects is the technique manual. These books describe the most commonly used techniques in a step by step manner. Some of the most commonly used manuals such as ***Molecular Cloning: a Laboratory Manual*** by Sambrook, Fritsch, and Maniatis are listed in the annotated references in the back of this book.

Trade and Technical Journals

Much of the information you may need will be found in trade and technical journals published by trade and professional associations. The publications may be found at university and industrial libraries and libraries of some federal, state and local government offices. A number of these journals are listed under periodicals at the back of this book.

Indices

Newspaper, magazine and journal indices give you access to a broad range of publications. Nearly every library will have the ***Readers Guide to Periodical Literature*** which allows you to find articles in popular publications. Often, the library will have a copy of the publication or the library may have a microfilm or microfiche copy for viewing and copying in a special reader. One of the most exhaustive indices is ***Current Contents***, a weekly publication which reproduces the "contents pages" of thousands of journals and provides subject and authors indices. The monthly ***Applied Science and Technology Index*** is also helpful.

Abstracts

Abstracts publications provide extensive listings of research reports and technical papers presented at meetings or published in scientific and technical journals. Although not always available in many local libraries, most colleges libraries have abstract publications on energy, the environment, chemistry, biology, physics, mathematics, social sciences, and technologies such as biotechnology. Each April ***The Ohio Journal of Science*** publishes nearly 350 abstracts of papers presented at the Annual Meeting of The Ohio Academy of Science. There are a number of abstracts publications that contain abstracts dealing with biotechnology issues. Some of these are:

-Aquatic Sciences and Fisheries Abstracts
-Biological Abstracts
-Biotechnology Research Abstracts
-Energy Research Abstracts
-Food Science Abstracts
-Genetics Abstracts
-Microbiology Abstracts
-Plant Breeding Abstracts
-Virology and Aids Abstracts

Vertical File

The vertical file, sometimes called pamphlet file, often contains useful information not found by other means. Here you'll find publications from state and Federal agencies, corporations, trade and professional groups. The materials usually are arranged by subject and filed in large folders.

Additional Sources of Information

Some Ground Rules

At the back of the book is a section that lists sources of information on biotechnology that will enable the student to gain additional, detailed information on biotechnology. Before encouraging students to contacts these sources or authors of the projects, it is imperative that teachers give students some ground rules.

1. Be sure that students have written proposals or research plans for their projects including specific problems or objectives. They should also be expected to write an hypothesis, spell out the methods and protocols that they will use, and list the materials and equipment needed.

2. Students should exhaust local library resources first. Too often students write or call corporations, government agencies and institutions without first "doing their homework".

3. Instruct students to write letters which request specific information and not everything under the sun. Two sample letters follow:

Unacceptable Letter

Dr. J.D. Smith
Dept. of Biology
XYZ University
University Town, OH

Dear Dr. Smith:

I am doing a science fair project on food biotechnology. I don't know anything about this subject except that it is important, so will you please send me everything you have on the subject.

I need to have the information next week, so would you please hurry?

Sincerely,

Bill Smith

P.S. My friend Sally is doing her project on guppies.
She asked me to ask you for information on guppies too.

This is an Unacceptable letter for the following reason:

1. No date.
2. No address for return of information to the student.
3. Incomplete address (no zip code).
4. Request is too general; asks for everything under the sun.
5. The student has not narrowed the topic.
6. Student has not exhausted local library resources first.
7. Response time (next week) is too short.
8. The student should not ask questions for someone else, especially when they are unrelated (guppies?).
9. The student did not sign the letter.

Acceptable letter

William Smith
123 Smith Street
Smithton, Ohio 12345
(419) 333-4444

March 15, 1993

Mr. J.D. Jones
Director of Public Information
XYZ Corporation
Anytown, OH 43215

Dear Mr. Jones:

I am doing a student project on the effects of salt concentration on the production of sauerkraut. I have exhausted the resources at my school library and the nearby college library. I have also corresponded with Dr. J.D. Smith at XYZ University.

Knowing that XYZ Corporation produces sauerkraut, I thought that you might be able to help me locate recent data on how variations in salt concentration influence the sauerkraut fermentation process. Do you have any recent reports or data which you could share with me? Is there someone at XYZ Corporation who would be willing to talk with me about this request?

To help you respond to my questions, I have enclosed my research plan and a summary of the information I have collected along with a list of the references I have used.

Thank you for your consideration of my request.

Sincerely,

William Smith

Enclosures

This letter is more acceptable for the following reasons:

1. Includes return address, phone number and date.
2. Student shows evidence of using local library resources.
3. Request is more specific than other letter.
4. Student is sending a description of the project and a summary of his background information which will help the person at the XYZ Corporation respond without duplication information already collected.
5. Shows that student is already familiar with corporation.
6. Student suggests that talking with someone may be good way begin a mentor relationship.
7. Student is polite ("Thank you for your...) and is not demanding that a response be sent by next week.
8. Student doesn't ask help for his friend.

DEVELOPING HYPOTHESES AND OBJECTIVES

Hypotheses

A successful science day or science fair project must have a testable or verifiable hypothesis. An hypothesis is a statement describing an expected outcome of an experiment. It is what the researcher expects to happen.

The following table outlines three problems or questions and suggests three corresponding hypotheses which may be tested.

Problem	Hypothesis
What is the influence of salt concentration on the production of sauerkraut	A concentration of 2.5% NaCl in chopped cabbage will produce the highest quality sauerkraut.
What is the influence of salt concentration on the microbial populations during sauerkraut fermentation	Low salt concentration will allow the growth of microbes that are not normally associated with sauerkraut fermentation
What is the effect of culture conditions on the production of amylase by *E. coli*	A low concentration of glucose and a high concentration of soluble starch will result in the highest production of amylase by *E. coli*

The Verifiable Objective

Although it is important from an educational perspective to always have the student develop a hypothesis, it may not always represent the way in which some biotechnology projects work, especially in the early stage of a project. For example, the problem may be to increase the production of amylase. A student may have hypothesized that the amylase gene from bacteria X would yield more product if it was expressed in bacteria Y. This means that the student will have to identify and isolate the amylase gene from bacteria X and clone it into a vector and then insert the vector into bacteria Y. All of this must be accomplished before the hypothesis can be tested. A student could spend a full year on each phase of the project, and yet it would not be until the third phase of the project that the hypothesis could be tested.

It is possible to say that the hypothesis for the first phase is that the amylase gene in bacteria X can be identified and isolated. However this seems a bit artificial and forced. When one reads the scientific literature, hypotheses of this type are not stated, except for those papers that are representative of the pioneering work in gene engineering. Because of the knowledge that is available to researchers and students, it seems more natural to state an objective as opposed to an hypothesis for a project such as phase one of the research described here. Testing must take place at each phase to ensure that the gene has been identified and that it has been isolated. Once cloned, testing must be done again to ensure that the gene has been inserted into the vector and that the vector, with the gene X still intact, has been inserted into bacteria B and only then will one be testing the original hypothesis of the project.

Most of the projects in this book will lend themselves to forming hypotheses, however in a few cases the statement of a verifiable objective may be more appropriate.

SUGGESTED STUDENT RESEARCH PROJECTS

I. Biotechnology in Food Production

II. Biopolymer Synthesis

III. Plant Biotechnology

IV. Enzyme Expression and Production

V. Altering Genetic Characteristics

VI. Applications of DNA Analysis

VII. Protein Analysis

VIII. Industrial and Environmental Biotechnology

IX. Cellular Physiology

BIOTECHNOLOGY

IN

FOOD PRODUCTION

FERMENTING ("DOUGH-RAISING") POWER OF BREAD YEASTS

KEY WORDS: Yeast, Food microbiology, Fermentation, Bread.

BACKGROUND:

Bread dough is usually leavened by bakers' yeast (actively gas-producing strains of *Saccharomyces cerevisiae*). Yeasts ferment the sugar in the dough, producing ethanol and carbon dioxide. CO_2 is the leavening agent; the alcohol evaporates off during baking. Sometimes other gas-producing microorganisms are involved in bread leavening; these usually are heterofermenting lactic acid bacteria (sourdough bread or salt-rising bread).

Strains of *S. cerevisiae* used in the manufacture of bakers' yeast are usually single-cell isolates that have been selected especially for the purpose. They should give a good yield of cells in the mash or medium chosen for their cultivation, should be stable in their characteristics, should remain viable in the cake or dried form for a reasonably long period during storage before use, and should produce carbon dioxide rapidly in the bread dough when used for leavening. See Chapter 20 in Frazier and Westhoff (1978).

Commercial yeast is prepared and sold in two forms: yeast cakes and active dry yeast. Yeast cakes contain, in addition to yeast cells, small amounts of starch, vegetable oils, and some lactic acid bacteria. Active dry yeast is made by drying the yeast cells to less than 8% moisture. Cells so dried are grown especially for the purpose and are dried carefully at low temperatures so that most of the cells will survive, and during storage at room temperature will retain for some months their ability to actively leaven dough. In recent years, active dry yeast has nearly supplanted yeast cakes.

We have observed in this laboratory that various commercial yeasts differ rather markedly in their "dough-raising" ability. It has also been observed that the effectiveness of dough-raising by a given yeast preparation is positively correlated with the percentage of living cells as shown by a wet mount of cells in a methylene blue solution. "Living" cells, by this criterion, maintain a permeability barrier whereas "dead" cells freely allow the entrance of the dye, and stain some shade of blue. The reason for the use of quotation marks around "living" and "dead" is that, by other criteria (such as ability to form a visible colony on solid media) a very few of the "living" cells might actually be "dead" - or vice versa.

STATEMENT OF PROBLEM:

This experiment will compare two brands of active dry yeast in their ability to raise dough and in the percentage of living cells, as shown by the methylene blue test.

METHODS AND MATERIALS:

1/ ea.	50 or 100 ml/graduated cylinder (to measure 30 ml distilled water)
1/4 stud.	100 ml graduated cylinder, greased with Vaseline
50 g/4 stud.	Flour
1 g/4 stud.	Two brands, A and B, of active dry yeast. (If desired, a yeast cake could be added, if obtainable in Brand A and/or Brand B.)
1/table	*Saccharomyces cerevisiae*, young streak culture (solid medium)
2/table	Square sheets of brown wrapping paper (24" x 24" or larger)
30 ml	Buffered methylene blue stain: Mix 1 part of 1:5,000 methylene blue & 1 part of a phosphate buffer solution (99.75 ml of 0.2 M KH_2PO_4 to 0.25 ml of 0.2 M Na_2HPO_4) to give pH of 4.6.

Procedure.

1. Examination of Yeasts:

Prepare wet mounts of the following yeasts:

a. Dry yeast

b. Young culture of *S. cerevisiae* on agar slant

Add 1 part buffered methylene blue to 3 parts yeast suspension, and cover with a cover slip. Examine immediately (Use the 10x objective first to focus and then go to the 43x objective).

"Living" cells should appear white, or almost white, and "dead" cells some shade of blue. Record the % of living cells for each yeast sample (estimated %). Count blue and colorless cells in 4 or 5 fields (depending on density of cell suspension).

	% "LIVING CELLS	
	BRAND A	BRAND B
YEAST CAKE		
DRY YEAST		
CULTURE		

2. Fermenting ("dough-raising") power of Yeasts:

a) Work in groups of 4 or 5. Weigh out 50 g flour on a piece of waxed or brown wrapping paper. Mix 1 g dry yeast, 30 ml distilled water, and enough of the flour to make a thin sponge. Then mix in the remaining flour and knead vigorously for 5 min. Roll the dough into a cylinder (greased with Vaseline), and press down. Read volume (should not be over 25-30 ml) Incubate at room temperature and read volume every 30 min, for 90 min. Record volume and % increase in volume (compared to initial volume).

b) Each group should set up 2 cylinders, one with the yeast brand A, and one with yeast brand B.

c) The whole class should share results in order to have more samples, and a better chance for detecting a significant difference between the samples.

STUDY QUESTIONS:

1. Were any differences observed in dough-raising power between the two brands of yeast? List them in the order of activity.

2. Was there a positive correlation between the percent of living cells and dough-raising power? The preparation with the higher percentage of living (colorless) cells should show the greatest increase in dough volume.

3. Put your results on the blank line or on a form provided by the instructor.

When everybody's results are in, copy them all down. Note the different values obtained by different people. Such variations in values nearly always occurs when measurements are made of complex biological systems (as this is). But the results vary around a certain value which could be called the true value.

Calculate the average from the whole class - of the results given for percentage of living and dead cells, in both brands A and B, and also the averages of the dough-raising power (increase in volume of dough in ml. 90 min.). If the differences in the average values for A and B are less than the largest difference between individual values for A or for B, the differences between A and B is probably not significant. These considerations are the basis for the methods of statistics, about which you will learn more later.

REFERENCES AND RESOURCES:

Boyd, Robert F. 1988. General Microbiology 2nd ed. Times Mirror/Mosby College Publishing, St. Louis.

Frazier, W. C., and D. C. Westhoff. 1978. Food Microbiology, Third Edition, McGraw-Hill, Inc., New York.

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FERMENTATION BY BACTERIA - FOOD PRODUCTION

KEY WORDS: Fermentation, Yogurt, *Streptococcus, Lactobacillus.*

BACKGROUND:

Crude techniques in biotechnology have been around for over 100 years. In 1876, Pasteur published a book on beer in which he explained the process of fermentation. Fermentation is one of the processes in food microbiology that shows the positive aspects of microorganisms. People of the Near East have used fermented milk products for centuries. Unknowingly, it was probably used as a preservative for the milk products. The acid produced killed the pathogenic microbes.

This exercise in yogurt production demonstrates fermentation by *Streptococcus thermophilus* and *Lactobacillus bulgaricus*. Heated milk is inoculated and maintained at a given temperature, while bacteria grow and ferment the milk sugar. The fermentation produces an acid which coagulates the milk protein. The final food product is a white, custard-like, acidic curd. The quality of the curd depends upon three things: (1) the starter culture, (2) the incubation temperature, and (3) the holding time. Proper starter volumes yield a firm curd. Longer heating times yield a firmer curd. Longer incubation times yield a more tart curd.

Other variables that can be manipulated are the flavors, types of starter strains, and the percentage of butterfat.

STATEMENT OF PROBLEM:

To demonstrate fermentation through yogurt production.
To determine the effect of holding time on the curd texture.
*To verify the organisms involved in the fermentation process.

(* refers to an optional extension to the basic exercise)

MATERIALS:

pint of milk sample
600 ml beaker
thermometer
pH paper (or meter)
pan of water
tripod and burner (or hot plate)
methylene blue stain
foil, teaspoon, and spreading rod
yogurt inoculum (Dannon plain)
slides and microscope
*3 sterile 1 ml pipets
2 sterile dilution blanks
1 poured LAB agar plate
Gram stains
candle jar (or CO_2 incubator)

METHODS:

1. Pour the milk sample in the beaker. Check the pH and record this, as well as milk type, in the Results.

2. Place the beaker in the pan of water and heat the milk to 80°-81°C. This is the holding temperature. Maintain for 5 to 30 min. Record your chosen holding time.

3. Cool the milk to 40°-41°C. Use the thermometer to obtain accurate readings.

4. Add 1 rounded tsp. of the yogurt starter. (see note 1) Stir with a clean spoon and cover with foil. If beakers are limited, pour mixture back into original carton and close securely.

5. Incubate at 40°C for 8-18 hours. Do not stir or disturb. (see note 2)

6. Check final pH. Refrigerate when firm.

7. Remove a small sample and put it on the end of a glass slide. Make a thin smear.

8. Air dry and stain with methylene blue for 1 min.

9. Carefully rinse with water and blot dry.

10. Examine under oil-immersion. Look for the different shapes of microorganisms involved. If the experiment ends here go on to 11. If you are continuing on to the extension do 13-15 before adding flavors and tasting.

11. Sugar, flavors, or fruit can now be added to the cooled curd.

12. Taste your product and record all findings in the Results.

Note 1-Dried starter cultures are available from Hansen's Lab Inc., 9015 W. Maple St., Milwaukee, Wis., 53214. Local health food stores may also carry dried preparations.

Note 2-If no incubator is available, there are alternatives....crockpot at lowest setting, pan of warm water placed in oven turned on for 10 min then off-put culture in pan and keep oven closed, styrofoam cooler with light bulb as heat source-watts can be calibrated to different temps.

*13. Remove 1 ml of the cooled curd and add it to dilution blank, containing 9 ml of sterile H_2O. (10^{-1})

14. Make one more dilution, as above by removing 1 ml from tube one and adding it to the second prepared blank. (10^{-2})

15. Pipet 1 ml from the final dilution onto the prepared petri plate and spread evenly. Incubate at 37°C for 3-4 days in a candle jar.

16. Pipet 1 ml from the final dilution onto the end of a glass slide. Prepare a smear and stain using the Gram stain procedure.

17. Observe 5 different fields, on your slide, under oil immersion. Count the number of clumps (colonies) of each bacterial type. Average your counts so that you have one average number for each bacterial type. Record these numbers in the Results.

18. After the incubation period observe your plate. The large, white colonies are the *Streptococcus* and the small clear colonies are the *Lactobacillus*. Count the colonies on the plate. Which type is seen most often? Record in the Results.

RESULTS

type of milk	beg. pH	hold. time	texture	taste	final pH	slide aver. # org.	plate colony #	gram st# aver.
						ROD	ROD	RED
						COCCI	COCCI	BLUE

Optional Extension

Different starter cultures might be obtained and the quality of the resulting yogurt can then be compared. The type of milk (skim, 2%, or whole milk) used could also be a variable.

REFERENCES:

Dubos, Rene. 1988. Pasteur and Modern Science. Science Tech. Inc. Wisc.

Hewitt, Jean. 1971. The New York Times Natural Foods Cookbook. Quadrangle Bk. pp. 310-313.

Rose, A.H. 1981. "The Microbiological Production of Food and Drink". Scientific American. Sept. 245:126-134.

Volk, W.A. and Wheeler, M.F. 1988. Basic Microbiology. Harper and Row. NY. pp. 601-610.

RESOURCES:

American Society for Microbiology (slides, written materials) 1913 I St. NW, Washington, DC 20006.

American Type Culture Collection (cultures, written materials) 12301 Parklawn Dr., Rockville, MD 20852.

Industrial Biotechnology Association (written materials) 2115 E. Jefferson St., Rockville, MD 20852.

Society for Industrial Microbiology (written materials) P.O. Box 12534, Arlington, VA 22209-8534.

These organizations will help students directly or lead them to another appropriate resource.

Agar-

Lactose	20 g	Tryptone	10 g
meat extract	10 g	yeast extract	10 g

Tween 80	1 g	K_2HPO_4	2 g
agar	15 g	filtered tomato juice	50 ml

water to 1 Liter adjust pH to 6.6

Difco handles the ingredients, testing and college labs may also be used as a source of materials as well as biological supply companies.

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THE SAUERKRAUT FERMENTATION

KEY WORDS: Sauerkraut, Fermentation, *Leuconostoc, Lactobacillus, Pediococcus.*

BACKGROUND:

Sauerkraut is an example of a fermented vegetable food which is produced through a natural fermentation; that is, microorganisms naturally present on the raw cabbage produce the desired changes resulting in the final product. Shredded cabbage is mixed with a quantity of salt and placed in a container in which anaerobic conditions can be achieved. The salt serves to extract liquid from the vegetable tissue so that it becomes available for the fermentation bacteria to grow in; the high salt content also acts as a selective agent so that the desired bacteria will be favored for growth. The first to grow are *Leuconostoc mesenteroides*, which produce lactic acid and carbon dioxide. As CO_2 accumulates in the product, near anaerobic conditions are achieved and this, along with the lowering of the pH due to lactic acid production, helps to inhibit growth of undesirable microorganisms. Later in the fermentation the more acid-tolerant lactic acid bacteria *Lactobacillus brevis*, *Lactobacillus plantarum*, and sometimes *Pediococcus cerevisiae* grow and contribute to the end products of the fermentation.

STATEMENT OF PROBLEM:

The purpose of this project is to determine the effects of altering environmental parameters on the sauerkraut fermentation.

METHODS AND MATERIALS:

The only materials which will be needed to actually set up the sauerkraut fermentation are fresh cabbage, sodium chloride, large (1 L) glass beakers, plastic garbage bags, and rubber bands. The fermentation is monitored by following acid development (either by pH determinations or titratable acidity), microbial succession (rods/cocci via Gram stain), and sensory determination (smell and/or taste). In general, the fermentation is set up in the following way: the cabbage heads are trimmed of dirty leaves, then cored. The cabbage is then finely shredded (1/16-1/32 inch size), after which NaCl is mixed in at a level of 2.25-2.5%. The shredded cabbage is immediately packed into the glass beakers (1/2-2/3 full), and a plastic sheet is placed on top of the beaker. The sheet should be large enough so that it can be placed flush against the top of the cabbage with enough left to drape over the sides of the beaker. Water is poured on top of the sheet so that it weights down the cabbage, then the sheet is secured to the beaker with a rubber band. The fermentation is then allowed to proceed for 1-2 months, until a total acidity of 1.7-2.3% is achieved.

In this project, the effects of varying initial NaCl concentration, different fermentation temperatures, and aerobic fermentation on the final product will be determined. For each parameter or variable investigated, the product will be sampled weekly by "sacrificing" the fermentation vessel. Therefore, for a fermentation which will be sampled weekly over a period of five weeks, five beakers must be prepared for each parameter. After the contents of a beaker are sampled, they are discarded. Suggestions for parameters which can be monitored include 0, 1.0, 1.5, 2.0, 2.5 and 5.0% added NaCl, incubation temperatures of 7.5°, 18.3° and 32°C, and anaerobic vs. aerobic fermentation. At the beginning of the fermentation, and at weekly intervals, check the cabbage juice for acidity, the types of microorganisms (by Gram stain), and the relative ratios of rods/cocci present. You should be able to observe the microbial succession in the fermentation under the standard conditions (2.5% NaCl, anaerobic at 18.3°C). Also visually inspect each sample and note the aroma and the taste. If the sample is obviously spoiled or otherwise dubious looking, don't taste it. The fermentation is over after no more acid develops.

This project should give you an understanding of what a food fermentation is and how microorganisms can bring about these fermentations. The importance of controlling environmental parameters in natural fermentations will become obvious after completion of the project. For those adventurous enough to attempt it, you may wish to isolate and purify the predominant microorganisms at the various stages of the fermentation, and confirm their genus identity. Two bacterial media useful for the isolation and growth of lactic acid bacteria are MRS and APT media, available from Difco Laboratories, Detroit, MI. Also, other vegetables may be used for fermentations; suggestions for producing other fermented vegetable products can be found in the literature.

Optional Extension

The freshness of the cabbage could be looked at as a factor influencing sauerkraut fermentation. A comparison could be made between the results when freshly picked fall cabbage is used as opposed to cabbage which had been in storage.

REFERENCES:

Anonymous. 1977. *Difco Manual of Dehydrated Culture Media and Reagents for Microbiological and Clinical Laboratory Procedures*, 9th ed. Difco Laboratories. Detroit, MI.

Gilliland, S.E., ed. 1985. *Bacterial Starter Cultures for Foods*, 4th ed. CRC Press, Inc. Boca Raton, FL.

Reed, G., ed. 1982. *Prescott and Dunn's Industrial Microbiology*, 4th ed. AVI Publishing Co. Westport, CT.

Speck, M.L., ed. 1984. *Compendium of Methods for the Microbiological Examination of Foods*. American Public Health Association. Washington, D.C.

Wood, B.J., ed. 1985. *Microbiology of Fermented Foods*, Vol. 1. Elsevier Applied Science Publishers. London and New York.

An additional reference for general microbiology techniques: Gerhardt, P. *et al.*, eds. 1981. *Manual of Methods for General Bacteriology*. Washington, D.C.: American Society for Microbiology.

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BIOPOLYMER SYNTHESIS

CELLULOSE PRODUCTION BY *ACETOBACTER XYLINUM*

KEY WORDS: Cellulose, Biopolymer.

BACKGROUND:

Cellulose is the most abundant biological polymer on Earth. It is synthesized primarily by green land plants, and since it is a renewable resource, there has been much interest in developing cellulose production for biotechnological purposes. Cellulose forms the basis of fibers that are used for clothing and even for the paper upon which this experiment is written.

The structure of the cellulose polymer is well-known. It is composed exclusively of repeating subunits made from glucose, a carbohydrate. Though the structure of cellulose has been well studied, little is known about the biological synthesis (biosynthesis) of this important molecule. The synthesis of cellulose needs to be better understood if there is to be commercial biotechnological use of this material. To this end, scientists look for model systems to study biological processes. To study cellulose synthesis, scientists have turned to a single-celled organism, a bacterium, that synthesizes cellulose as part of its normal metabolic activity. Bacteria have been widely used in biotechnology because of their small size, yet prodigious reproductive rates, diverse metabolic capabilities, and ability to be manipulated genetically. These are reasons why the study of cellulose synthesis by a bacterium is of interest. *Acetobacter xylinum* is the name of the microorganism that synthesizes cellulose. The glucose subunits that comprise the cellulose polymer are extruded through pores in the surface of the bacterium. The cellulose fibirils form into a mat or pellicle within which the bacteria are held at the air-liquid interface when the bacteria are cultured in the laboratory. In other words, the pellicle floats to allow the bacteria plenty of air, which they require in order to grow, multiply, and synthesize more cellulose.

STATEMENT OF PROBLEM:

To measure the effects of variation of culture media constituents upon the production of cellulose by *Acetobacter xylinum*, a cellulose-synthesizing bacterium.

METHODS AND MATERIALS:

Note - When working with bacteria and microbiological materials, aseptic (sterile) techniques must be used in order to avoid contamination by unwanted microorganisms.

Bacterium - Cellulose-synthesizing strain of *Acetobacter xylinum* (available from the American Type Culture Collection - ATCC 23769). This bacterium is not pathogenic, but good aseptic techniques should be practiced when handling it. **NO MOUTH PIPETING!**

Microbiological Medium : Glucose - 20 g/l; Yeast Extract - 5 g/l; Peptone - 5 g/l; Sodium Phosphate - 2.7 g/l; and Citric Acid - 1.15 g/l. When making solid medium to culture the bacterium in petri dishes, add agar at 15 g/l. Prepare medium using distilled water.

Methods - Prepare medium and dispense into tubes large enough to hold 10 - 15 ml. Sterilize the medium using an autoclave or pressure cooker. (Note - tubes of the medium can be used for culture and storage of the bacterium, or agar slants in screw-capped tubes can be used for long-term storage of bacterial cultures.) After the medium has been sterilized and cools, inoculate it with a defined amount of bacterial culture (1 ml, 2 ml, 0.5 ml, 0.1 ml, 1 drop with a sterile Pastuer pipet, 2 drops, 5 drops). Then carefully pour the contents of the tube into a sterile Petri dish. Incubate the dish at room temperature (or in an incubator up to 30°C, which is the optimum temperature for growth of *Acetobacter xylinum*). Observe the medium daily to watch for the cellulose pellicle to form. After a week of incubation, carefully lift out the cellulose pellicle with a bent glass rod. Touch it. Let the excess liquid drip from it.

Weigh it on a good balance to determine its wet weight. Dry it in an oven for a few hours, and weigh it again to determine the dry weight. Describe what the cellulose looks and feels like. Grow the bacterium in the same way, but vary the concentration of glucose and/or the other constituents in the medium to measure the effects upon cellulose production. Test other carbohydrates such as fructose, sucrose, mannitol, or sorbose. Try some other organic carbon sources such as ethanol instead of glucose. Omit some of the ingredients of the medium to measure their effects upon cellulose production or the growth of the microorganism.

REFERENCES:

Brown, R.M., Jr. (ed) 1982. Cellulose and other Natural Polymer systems. Plenum Press, New York, NY

Forng,E., S. Anderson, and R. Cannon. 1989. Synthetic medium for *Acetobacter xylinum* that can be used for Isolation of Auxotrophic Mutants and Study of Cellulose Biosynthesis. Applied and Environmental Microbiology 55:1317-1319.

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BIOPOLYMER SYNTHESIS FROM SUCROSE WITH THE ENZYME DEXTRANSUCRASE FROM *LEUCONOSTOC MESENTEROIDES* NRRL-B512F

KEY WORDS: Leuconostoc, Biopolymer, Dextransucrase, Dextran, fermentation.

BACKGROUND:

Dextran is a glucose polymer joined mainly by α(1-6) glucosidic bonds when produced by *Leuconostoc mesenteroides* dextransucrase. It has an important place among the extracellular polymers produced by biological processes. Dextransucrase from the NRRL-B512F strain has been extensively studied. Unlike other strains, it produces dextran with only 5% branching in α(1-3). The enzyme is released when the strain grows in a defined medium (Lopez and Monsan, 1980). It is recovered by centrifugation of the biomass and can be purified by liquid-liquid extraction with polyethylene glycol. As sucrose is needed in the fermentation medium as an enzyme inducer, a certain amount of dextran is produced during fermentation

At present, two different alternatives exist for dextran production: by direct fermentation, that is enzyme synthesis and enzyme reaction taking place at the same time during fermentation, or by enzymatic synthesis: the reaction takes place with the enzyme separated from the cells, with or without further purification. Enzyme immobilization is an alternative that could be explored and compared with the soluble enzyme process.

If an optimal enzymatic batch reactor is to be designed for dextran production with the soluble enzyme, several parameters require a close examination. A compromise between enzyme concentration and reaction time is needed, as enzyme is expensive and so is labor. Substrate concentration cannot be as high as one would wish as the enzymes are subjected to excess substrate inhibition. Reaction parameters are also to be defined: pH can be fixed between 5.2-5.4, but reaction temperature definition requires a close look into enzyme stability. Finally, one has to decide if the enzyme is used as obtained after centrifugation or if a concentration-purification step is required.

STATEMENT OF PROBLEM:

The objective of this problem is to determine the optimal batch reaction conditions for the enzymatic synthesis of dextran. There are many parameters that could be investigated, such as batch volume, substrate concentration, reaction temperature, enzyme activity, and reaction time. The proposed kinetic model of Martinez and Lopez-Munguia (1985) could be used as a basis for the investigation.

METHODS AND MATERIALS:

Fermentations can be carried out in proper fermentors or, if not available, in agitated flasks. The strain of *Leuconostoc mesenteroides* NRRL-B512F can be obtained from the NRRL in Peoria, IL, but a microbiology laboratory should take care of the initial steps in order to recover the dried strain.

Culture medium: Sucrose 20 g/l, yeast extract 20 g/l, K_2HPO_4 20 g/l, $MgSO_4 \cdot 7H_2O$ .02 g/L and $MnSO_4 \cdot H_2O$, NaCl, $FeSO_4 \cdot 7H_2O$ .01 g/l; pH 6.0 and 29°C.

The fermentation can be followed by measuring the optical density at 650nm with a spec 20.

Enzyme activity measurement can be accomplished by following the release of fructose with a reducing sugar measuring method, during the first 10 min of reaction with the enzyme in acetate buffer pH 5.2-5.4, 30°C and 10% sucrose.

The reaction can also be followed by measuring the increase in viscosity as dextran is produced.

The produced polymer can be recovered by ethanol precipitation (2 volumes of ethanol per 1 volume of reaction medium), filtration and drying.

The enzymatic reactions can take place in stirred vessels with temperature regulation.

Other equipment needed are: balance, pH meter, incubators, autoclave, spectrophotometer, centrifuge and reaction vessels.

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PLANT BIOTECHNOLOGY

PLANT GENETIC ENGINEERING

KEY WORDS: *Agrobacterium*, Opines, Crown gall, Plasmid, Tumors.

BACKGROUND:

One method of plant genetic engineering utilizes a pathogenic system found in nature. This system involves the bacterium *Agrobacterium tumefaciens* which causes the disease, crown gall. *A. tumefaciens* infects many plants, noticeably dicots such as tobacco, by entering the plant at a wound site and causing an overgrowth of cells to appear. This overgrowth resembles a tumor and cells taken from this tumor have acquired new characteristics. When tumor cells are placed in tissue culture in the absence of the bacteria, the tumor cells will grow in the absence of the plant hormones, auxin and cytokinin, whereas normal plant cells require these hormones. In addition, these tumor cells produce novel metabolites such as octopine and nopaline (opines), which are not produced by normal cells.

The genetic engineering of plants comes about because of the acquisition of new characteristics by the plant as a result of the bacterial infection. The bacteria has a plasmid DNA molecule with the genes for these characteristics. During the infection a fraction of this plasmid is transferred to the plant and this plasmid DNA is integrated into the plant DNA. The plant has now been genetically engineered by the bacterium to produce novel metabolites and to grow in the absence of hormones. Genetic Engineers take advantage of this knowledge by removing much of the bacterial portion of the plasmid which will be inserted into the plant and replacing it with a gene of interest. This technique can serve as a mechanism of inserting genes which give this plant desired characteristics such as insect resistance and herbicide resistance.

STATEMENT OF PROBLEM:

To demonstrate tumorigenesis or the ability of *A. tumefaciens* to form tumors on plants.

MATERIALS:

1. Young sunflower seedlings growing in vermiculite.
2. *Kalanchoe daigremontiana* (maternity plant).
3. Liquid culture of *Agrobacterium tumefaciens* strain ATCC 6955 (an educational strain). A plate culture of this bacteria can also be used. All other strains of *Agrobacterium* are unavailable to students and teachers unless they have a permit to handle them.
4. Toothpicks and/or Pasteur pipets.

METHODS:

1. Puncture the sunflower with the toothpick just below the cotyledons. If using *Kalanchoe*, use the toothpick to make a trough on a young leaf or to puncture the stem just below the youngest leaf.
2. If using a liquid culture, pipet the bacteria and drop them onto the wound site. If using a plate culture, use a toothpick to scrape off some cells and paste them onto the wound site.
3. Make sure at least one plant is wounded and **not** inoculated. This is the control.
4. Incubate 2-4 weeks in the greenhouse or near a window.
5. Note the size and shape of the tumor.

Other plants that can be inoculated include tobacco, petunia, carrot disks, potato disks, and tomato. Try other plants and see whether they work as well as some of the plants suggested. Is the site of inoculation important to formation of the tumor?

Agrobacterium is a plant pathogen and thus must be used and disposed of carefully. At the end of the experimentation, all cultures should be sterilized by autoclaving at 121°C for 15 min

and all plants should be disposed of by incineration.

RESOURCES:

Seed for plants are available from most seed order companies such as Burpee, Park, or Harris seed. *Kalanchoe daigremontiana* can usually be found in stores that sell houseplants. These plants can be easily propagated asexually from the baby plants that grow off the leaves (hence the term maternity plant or mother of a thousand babies). *Agrobacterium tumefaciens* is available from the American Type Culture Collection and Carolina Biological Supply Company. Many different strains are available. Try them on different plants to determine whether the strain used causes variation in tumor formation.

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ISOLATION OF MORPHOLOGICAL MUTANTS OF THE HIGHER PLANT *ARABIDOPSIS THALIANA*

KEY WORDS: *Arabidopsis*, Mutation.

BACKGROUND:

There is substantial evidence that the diversity of life observed on Earth has arisen through the process of evolution. Evolution is thought to act by natural selection, a process in which individuals that are most suited for survival in a particular environment tend to have a reproductive advantage. Within a species, this "selection" operates on the natural variation within a population, which is predominantly due to slight differences in the genetic makeup of these individuals. One major mechanism that causes genetic variability within populations is the process of mutation. Mutations are heritable changes that arise by the modification of the DNA molecules which are responsible for passing genetic information from one generation to the next. Mutations can be caused by environmental factors such as X-ray and gamma-ray irradiation and exposure to a variety of chemicals.

One approach that plant breeders have used to try and improve the characteristics of agriculturally important plants is to speed up the process of evolution by purposely causing mutations in populations of plants and looking for mutations that exhibit desired characteristics. More recently, this approach has been used to identify plants that are resistant to certain herbicide treatments. This exercise will introduce the concepts of mutagenesis and mutant identification through mutant screens and mutant selections. In this exercise, you will work with a plant called *Arabidopsis thaliana. Arabidopsis thaliana* is a small weed which is related to plants in the mustard family. This plant, because of its rapid growth cycle and small genome size, has proven to be an excellent laboratory organism for both classical and molecular genetic studies of plant growth and development.

STATEMENT OF PROBLEM:

The purpose of this exercise is to identify plants with mutant phenotypes in a mutagenized population of *Arabidopsis thaliana* and to determine the genetic basis of the mutation.

METHODS AND MATERIALS:

Wild type and mutagenized seeds of *Arabidopsis thaliana* are available from a number of plant science departments at research universities throughout the country. If seed is not locally available, it may be purchased from Lehle Seeds. Also, students may wish to develop their own mutagenesis protocols to test the mutagenic capability of various chemicals or environmental stresses. However, if this approach is taken, it should be done under supervision. Seeds are sown on soil mixtures (MetroMix 200 or equivalent) and can be grown under lights on a table or shelf at room temperature. The seeds, which are very small, are sown by suspending approximately 200 seeds (5 mg) in 10 ml of a 0.1% agar solution. The evenly suspended seeds are then dispersed with a pipet onto a flat containing wetted soil and placed under fluorescent plant lights. If placed under continuous light, the plants will go through a complete life cycle in 4 to 6 weeks. For mutant screens, plant at least 10 flats. Be sure to plant some wild type seed so that comparisons can be made with the mutagenized plants.

After the seeds have germinated and begin to grow, the students should examine the plants for morphological differences between the normal and mutagenized plants. The types of mutations that have been observed by others include changes in leaf shape or number, the color of the leaves, the timing of flower development, the number of flower stalks that develop, and the shape of the flowers themselves. The students can devise more sophisticated screens such

as looking for mutations in the phototropic or gravitropic responses. Students can also devise *selections* for desired mutations. For example, it may be useful to identify plants that can grow in the presence of a herbicide or high salt concentrations. These experiments will require more work, since the minimum inhibitory concentration of the selective agent will have to be determined prior to the actual mutant selection.

Mutant plants should be carefully transplanted to a small pot and allowed to self-pollinate and set seed. When the seed pods begin to turn brown and dry out, carefully excise the pod from the flower stalk and place in a labeled vial. Allow the seeds to dry for two weeks; these are the M3 generation. Plant the M3 seed and observe the percentage of these plants that exhibit the mutant phenotype. Discuss what this result means in terms of the number of mutant alleles in the M2 parent.

Further analysis of the mutants can be done by crossing the mutant to a wild type plant (backcross). By backcrossing the mutant with a wild type plant, one can determine if the mutant phenotype is caused by single or multiple mutations, and if it is a single mutation, whether the mutation is dominant or recessive. This procedure is somewhat difficult with *Arabidopsis* because of the small size of the flowers and the fact that self-pollination normally occurs before the flowers open fully. To cross one *Arabidopsis* with another, find flower buds on one plant which have not yet opened and only the tips of the white petals are visible. At this stage, the stigma is receptive to pollination, but the pollen has not yet matured. Carefully remove the petals and the stamens from the flower with a pair of fine forceps (the use of a dissection scope or magnifying lens is strongly recommended). Next, carefully remove stamens from a fully opened flower on the plant with which you want to make a cross, and brush the pollen on the stamen onto the exposed stigma from the other parent. If you have been successful, a seed pod should develop in 1-2 weeks. Be sure to use the M3 plant as both a donor and recipient of pollen; some mutations cause the pollen to be sterile, thus the mutant can only be used as a recipient for pollenization.

The resulting seeds are called the F1 progeny. Plant these seeds and determine the ratio of progeny which exhibit the mutant phenotype. Allow the F1 progeny to self-pollinate, and collect the seed from individual plants. This is the F2 generation. Plant the F2 seeds and determine the ration of wild type and mutant individuals. Evaluate the results obtained with the F1 and F2 generations using Mendal's Laws.

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Suzuki, D., A. Griffith, J. Miller and R. Lewontian. 1989. An Introduction to Genetic Analysis. 4th Ed. W.H. Freeman, New York.

RESOURCES:

Lehle Seeds, 6531 N. Camino Katrina, Tuscon, AZ 85718 (602)554-0733.

Crucifer Genetics Cooperative, Dept. of Plant Pathology, 1630 Linden Drive, University of Wisconsin, Madison, WI 53706 (608)262-8638.

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INFLUENCE OF EXPLANT ORIGIN ON ADVENTITIOUS SHOOT REGENERATION IN *BRASSICA OLERACEA*

KEY WORDS: *Brassicca*, Tissue culture, Peduncle, Adventitious shoots.

BACKGROUND:

Although many different kinds of plant parts (roots, stems, leaves, flowers, etc.) can be placed in culture to produce adventitious shoots, the response of the different parts can be highly varied, depending on such factors as the culture medium, the species, and the age and health of the plant. The process of plant regeneration *per se* is extremely useful in propagating desirable plants, as well as in genetic engineering where the cells of the plant part that will form adventitious shoots become recipients of genes derived from bacteria such as *Agrobacterium*.

PROBLEM:

To determine the effect of the explant position within the plant on the subsequent production of adventitious shoots in the one type of medium and to infer about the physiological state of cells that respond to stimuli provided by the medium.

METHODS AND MATERIALS:

Rapid Cycling *Brassica oleracea* seeds.
potting mix
fertilizer
labels
4-in pots
growth chamber, light banks, or greenhouse space
Clorox
dish detergent
95% ethanol
scalpel & blade
forceps
paper towels wrapped in aluminum foil and autoclaved
sterile water
tubes or petri dishes containing MS medium, 2 mg/L Benzyladenine, 3% sucrose, 1% agar
Saran Wrap or Parafilm^{R}
clean area of a lab or laminar flow hood

The plant material recommended for this study is Rapid Cycling *Brassica oleracea* (RCBo), a relative of cabbage, broccoli, and cauliflower. Seeds of RCBo can be obtained from Crucifer Genetics Cooperative, Dept. of Plant Pathology, 1630 Linden Dr., University of Wisconsin, Madison, WI 53706. Seeds must be sown on fresh potting mix (e.g., Terralite RediEarth, Vermiculite). Five or six seeds can be sown per 4-in pot and 4-5 pellets of Osmocote^{R} complete fertilizer are added for every seed. The plants should be grown under continuous, bright light (as in a growth chamber) or else in a clean greenhouse. If kept under continuous illumination, the plants will begin to flower in approximately 3 weeks. The tissue of RCBo best suited for this experiment is the **peduncle**, the flower stalk. It is best to use peduncles just after the first flower in the inflorescence has opened, although older peduncles can also be used.

The major goal of the work is to determine which part of the peduncle produces the most adventitious shoots in culture. To achieve this goal, the student must keep track of the "upper" (the flower end) and the "lower" (the stem/leaf end) portions of the peduncle.

Steps to follow:

1. Obtain peduncles from 5 or 6 plants and mark the "upper" end by making a diagonal cut, and the "lower" end by making a perpendicular cut.

2. Wash the peduncles thoroughly with soapy water, being careful not to bruise them. Rinse in tap water to remove soap.

3. Place the peduncles in 100 ml of 15% Clorox (i.e., 15 ml Clorox + 85 ml water) in a very clean beaker. Add a small squirt of dish detergent. Place the peduncles so they are completely immersed in the solution, and keep them in the sterilizing solution for about 10 min; gently swirl the solution every other minute or so.

4. After the 10 min, use sterile forceps (some which have been kept in 95% ethanol for at least 30 min and then air dried in a clean, sterile area or laminar flow hood forceps may also be flamed off) to remove peduncles into another container having sterile water. Make sure the water and the container are sterile. Change the sterile water 3 times to remove all remaining clorox solution from peduncles.

5. Take a sterile bunch of paper towels and moisten with sterile water. Place a sterile peduncle on the moist towel while others remain in the water. With a sterile scalpel, cut cross-sections of the peduncle, each about 3-5 mm thick, starting from the top (the end with the diagonal cut). Keep each piece in its proper relative position. Assign a number to each piece starting from the top and give a letter to each peduncle. Thus, the uppermost 3 pieces of the first peduncle would be A-1, A-2, A-3, etc.

6. Place each piece on MS medium either in tubes or in petri dishes. If using a petri dish, place no more than 10 pieces per dish and write the number of each piece underneath it on the bottom of the plate. If using tubes, place one piece per tube and write the number on the side. Seal the plates either with parafilm or Saran Wrap. Place in a clean area in the dark for 2 days and then transfer to an illuminated shelf or bench.

7. Observe the explants every week and keep records in a journal of any changes that occur. After about 2-3 weeks, the student should be able to count individual shoot buds per explant. Plot the shoot number versus the position of the initial explant on the peduncle.

Modifications:

Numerous modifications of this basic experiment are possible. Each student may come up with some interesting ones like these:

1. Keep track of the position of the explant on the peduncle as well as its orientation. Does it make a difference if the sections of peduncle are placed on the media right side up, upside down, or on their side?

2. Consider different parts of the peduncle. Make longitudinal cuts instead of perpendicular cuts. When cutting lengthwise, cut the outer perimetes (the epidermal tissue) from the interior tissue (the core).

3. Consider modifying the hormone composition of the medium. Try 0, 0.2, 1, 2 mg/L BA. Also try with and without an auxin, eg., Napthaleneaceticacid (NAA) at similar concentrations.

4. Vary the environmental conditions. Is light required for adventitious bud formation? Does it make a difference if you use blue or red light?

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THE PRODUCTION OF PIGMENTS BY PLANT CELL CULTURES

KEY WORDS: Cell culture, Auxin, Cytokinin, Plant-derived chemicals.

BACKGROUND:

By using appropriate plant growth hormones, plant cells can be stimulated to grow in an undifferentiated mass, or callus, rather than producing the typical plant morphology. These undifferentiated cells can be readily grown under laboratory or industrial conditions, and thus should provide an alternative source of plant products to conventional agriculture. Particularly for tropical species where agricultural problems can exist, there is thus an interest in the exploitation of plant cell cultures.

Not all conditions which stimulate the formation of callus are necessarily favorable to the production of specific plant products. High levels of auxin and synthetic auxin-like chemicals are generally good for cell growth, but depress productivity. The exact nature of the hormones used, as well as their levels, can also affect product formation. Moreover, cell cultures are rarely homogeneous, with some cells reacting differently to others. Thus there is a need to experimentally determine the best conditions, and to select good cell lines from among mixed populations, in order to obtain high levels of product formation.

STATEMENT OF PROBLEM:

To determine optimal hormonal conditions for the production of selected pigments by plant callus cultures, and to select out possible high-producing lines. (Pigments are investigated here because they are easy to detect by eye.)

METHODS AND MATERIALS:

Suitable pigment-producing species include red beet (*Beta vulgaris*), garden varieties of *Berberis* or *Thalictrum* (care - the yellow pigments in culture and in the roots of intact plants are alkaloids which are toxic), Madder (*Rubia tinctorum*), safflower (*Carthamus tinctorius*), etc.

Pieces of root, stem, or more commonly, leaf are surface sterilized by immersion in 5-10% hypochlorite bleach for 20-30 min, followed by thorough washing with sterile water. The explant material is placed in jars on a sterile agar (8-10gm/L) gel containing 30g/L sucrose, nutrients and various combinations of auxin-type and cytokinin-type plant hormones. Murashige and Skoog's or Gamborg's nutrients are most conveniently bought commercially - e.g. Sigma Chemical Company, P.O. Box 14508, St. Louis, MO 63178, telephone 314-771-5750 - but can be made up. (For composition, see plant culture text books or contact Dr. A.J. Parr.) Suggested auxin-type hormones include indole acetic acid (the natural auxin), napthalene acetic acid, and 2,4D (care needed here, but the small quantities involved should not present a hazard if supervised correctly). Cytokinin-type hormones include benzyl adenine, kinetin and zeatin. Effective concentrations will probably be in the range of 0.1-2mg/L. After a few weeks, callus will appear on the cut surface of the explant. When well established, callus can be transferred to a range of further agar media and examined for pigment production. If any calli are obviously heterogeneous, pale and well-colored segments may be transferred separately to new media in order to develop separate lines which may ultimately result in stable low-producing and high-producing calli.

All solutions must be sterile, and all manipulation must be carried out aseptically in order to avoid bacterial and fungal contamination. This will require an autoclave and a laminar air-flow bench. These should be available in local plant or microbiological research institutes, hospitals and public health laboratories.

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Further help of both a general and more technical nature is available from Dr. A.J. Parr, Institute of Food Research, Norwich NR4 7UA, U.K. Possible local contacts include the Ohio State University Biotechnology Center, Room 206, Rightmire Hall, 1061 Carmack Road, Columbus, OH 43210, and Agrigenics Corporation, 35575 Curtis Blvd., Ste. 300, Eastlake, OH 44094.

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USE OF POLYACRYLAMIDE GEL ELECTROPHORESIS TO DETECT CHANGES IN PROTEINS AND PEROXIDASES IN MECHANICALLY INJURED MAIZE LEAVES

KEY WORDS: Peroxidase, Stress, Polyacrylamide, Isoenzyme, Electrophoresis, Electroblotting

BACKGROUND:

When higher plants are exposed to environmental stress or mechanical injury, a number of changes occur in enzymes and other proteins in the affected cells and tissues. Of the many enzymes whose activities are increased in response to mechanical injury, peroxidase is probably one of the most extensively studied. Peroxidase is one of the oldest known enzymes, is widely distributed among plants, and is readily extracted from plant cells and tissues. Also, peroxidase occurs as different isoenzymes whose levels vary in accordance with the severity and duration of mechanical injury. Moreover, some of these peroxidase isoenzymes have been implicated in the enhanced lignification which inevitably occurs following mechanical injury of plants. Thus, peroxidase is an effective model system for studying the molecular biology of plants.

STATEMENT OF PROBLEM:

The purpose of this project is to use various techniques of biotechnology to (1) determine the changes that occur in distinctive proteins and peroxidase isoenzymes from maize leaves at different time periods following mechanical injury and (2) estimate the extent to which the changes in peroxidase activity are due to altered enzyme synthesis versus activation or inactivation. The project is divided into several parts: (1) mechanical injury of leaves, (2) homogenization to obtain a protein extract, (3) separation of these proteins by electrophoresis on duplicate polyacrylamide gels, (4) staining of one gel to visualize proteins, (5) placing the other gel in an assay mixture to visualize peroxidase isoenzymes, and (6) if necessary, using electroblotting to transfer proteins to nitrocellulose paper for a more effective assay of peroxidase.

METHODS AND MATERIALS:

A. Preparation of Maize Leaf Extracts

1. Cut 16 leaf pieces (10 cm long) from leaves of comparable age from 3 or 4 week-old maize plants grown in standard potting soil in a greenhouse, growth chamber or other suitable location. Mechanically injure 8 of these pieces by gently rubbing the surfaces with carborundum, then rinsing with double distilled water. Rinse the remaining 8 leaf pieces with distilled water, but do not injure; these pieces will serve as controls.

2. Immediately place 4 injured and 4 control leaf pieces in a freezer until ready for use. Incubate the remaining pieces (4 injured and 4 controls) at 28°C in a water-saturated atmosphere in the dark for 48 hours, then store in the freezer as well. You should now have four groups of leaf pieces frozen and ready for analysis: (a) leaves injured and frozen immediately, (b) leaves uninjured and frozen immediately, (c) leaves injured but incubated 48 hours before freezing, and (d) leaves uninjured but incubated 48 hours before freezing.

3. To prepare extracts for electrophoresis, homogenize each group of 4 leaf pieces in 5.0 ml of 10 mM Sodium Phosphate buffer, pH 6, by thoroughly grinding with a mortar and pestle and 0.5 g of washed sand. Then centrifuge each of the resulting extracts at 4000 g sufficient to sediment particulate matter. Save the supernatant from each extract (they can be frozen, if necessary) for electrophoretic separation of peroxidase isoenzymes and other proteins.

B. Electrophoresis Equipment and Supplies

1. Gels. An easy and rapid method for separating and identifying proteins involves the electrophoresis of samples in miniature polyacrylamide gels (mini-gels). These gels can now be purchased (e.g. from Jule, Inc., 25 Science Park, New Haven, CT 06511) pre-cast with preformed sample wells and ready to use. We have obtained best results with 10-20% gradient, nondenaturing polyacrylamide gels, 0.75 micron in thickness; and we recommend that these gels be purchased for this project.

2. Electrophoresis units, electrotransfer apparatus and power supplies. Although there are several electrophoresis units for running mini-gels, we have used the Bio-Rad Mini-PROTEAN II™ dual slab gel (Bio-Rad Laboratories, P.O. Box 708, 220 Maple Avenue, Rockville Center, NY 11571). The electrotransfer apparatus compatible with the Bio-Rad unit is the Bio-Rad Mini Trans-Blot Module. Any power supply designed for electrophoresis and capable of generating 200 volts is also required.

C. Electrophoresis

1. Wearing gloves, unpack 2 pre-cast mini-gel "sandwiches" with comb-in-place and fit them into the Bio-Rad electrophoresis unit as indicated by the manufacturer.

CAUTION: The gels may contain some unpolymerized acrylamide which is a neurotoxin, and thus plastic disposable gloves are mandatory. In addition, commercial gels often contain the toxic agent sodium azide as a preservative. Thus, do not eat or drink in the laboratory, and as with all laboratory procedures, safety glasses are recommended.

2. Prepare a "running buffer" of the same composition as the buffer, in the commercial gels as indicated by the manufacturer. Also prepare a "sample buffer" which is a 1/4 dilution of the running buffer and contains 20% glycerol and 2 mg bromphenol blue per 100 ml total volume.

3. Slowly and carefully pull the attached comb straight out of the top of the gel and immediately use a disposable pipet to flush the resulting wells with running buffer.

4. Dilute 0.5 ml of each sample with 0.5 ml of sample buffer and mix well. If desired, these diluted samples can be frozen for use at a later time. Using a micropipet, carefully pipet aliquots of the diluted samples into the bottom of separate wells. BE CAREFUL not to mix the samples or contaminate other wells. To increase the chances of success, we suggest using a range of volumes (e.g. 10 and 20 μl) of each sample in separate wells. Prepare each of the two gels in exactly the same manner, and make careful note of the contents of each well.

5. Following the manufacturer's instructions, assemble the electrophoresis unit, attach the power supply and electrophorese at 200 volts for 45 min. Then (wearing gloves) carefully remove the gels and transfer to separate staining solutions, as described below.

6. Stain one gel for protein using either Coomassie Blue or silver nitrate stains as described in Davis *et al.* (1986).Stain the other gel for peroxidase by placing in a tray containing 100 ml Sodium Phosphate buffer (pH 5.5), 15 ml 50 mM guaiacol and 2.5 ml 100 mM H_2O_2 (use a fume hood). Depending on the sample, reddish-brown bands should appear at least within 30 min. If no bands appear, add 15 ml more of guaiacol and 2.5 ml more of H_2O_2 and incubate 30 additional min. If bands still do not appear, remove the gel, wash 15 min in distilled water and proceed to the electrotransfer step (Part D).

D. Electrotransfer (sometimes called electroblotting)

1. Following the manufacturer's instructions, electrotransfer the proteins from the gel to 0.45 micron nitrocellulose paper.

2. Once electrotransfer is complete, wear gloves to carefully remove the nitrocellulose paper, orient it so the side that faced the gel faces upward, and let it air dry. Then dip the paper in the assay mixture for peroxidase as outlined in step C.6.

ANALYSIS OF RESULTS:

Compare the results obtained with the two gels. The gel stained for protein will give an approximation of the many proteins present in the various samples. The gel (or nitrocellulose paper) reacted with guaiacol plus H_2O_2 will indicate which of the many protein bands correspond to peroxidase. By comparing the intensity of the staining, determine if wounding the leaves had any effect on either the levels of protein or peroxidase. Did the 48 hour incubation time have any effect? Look to see if new protein bands are visible as a result of the various treatments.

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RESOURCES:

The electrophoresis apparatus necessary to carry out all phases of this project can be purchased for approximately $1500. Pre-cast gels cost about $120 per box of 8. Chemicals and other expendable supplies will cost approximately $100.

The timing for carrying out this project is approximately as follows:

Preparation of stock solutions	1-2 hr
Electrophoresis	1 hr
Staining: Coomassie Blue	1.5-3 hr
Silver Nitrate	Day 1 - 1 hr
	Day 2 - 2 hr
Electrotransfer and staining	2 hr

It may be convenient to perform the electrophoresis and do the staining on the first day. Then, if necessary, the electrotransfer can be performed one or two days later, as long as the gel is stored in distilled water.

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PRODUCTION OF ALFALFA ARTIFICIAL SEEDS

KEY WORDS: Embryo, Clone, Callus, Hydrogel, Seed coat.

BACKGROUND:

A potential method for rapid, large-scale, vegetative propagation of plants was identified in 1958 when somatic (asexual) embryos were first produced in carrot (Steward *et al.* and Reinert). Since then, somatic embryogenesis has been observed in many species (Ammirato 1983). Somatic embryogenesis appears to be the only clonal propagation system suitable for crops currently propagated by seeds, but commercial application has not yet occurred. The limitations for a somatic embryo delivery system are: 1) coating systems have not been optimized; and 2) quality of somatic embryos is relatively poor.

Development of artificial seeds requires sufficient control of somatic embryogeny from initial explants, to embryo growth and development, to embryo maturation. Embryos must be capable of germinating out of the capsule or coating to form vigorous, normal plants (conversion). Commercial development of artificial seeds requires a somatic embryogeny protocol that results in mature embryos which will convert to normal plants.

To create a low-cost, high-volume propagation system, sufficient methods for producing high-quality somatic embryos and encapsulation of the embryos are required to allow for direct planting using existing farm machinery.

STATEMENT OF PROBLEM:

To produce high quality alfalfa somatic embryos and encapsulate them in calcium alginate beads to produce artificial seeds.

METHODS AND MATERIALS:

Alfalfa Somatic Embryogeny and Conversion

An excellent somatic embryogeny system has been developed for alfalfa, *Medicago sativa* L. (Fujii *et al.* 1988; Redenbaugh *et al.* 1986, 1987ab, 1988a; Strickland *et al.* 1987; Stuart and Strickland 1984ab; Stuart *et al.* 1985ab, 1987; Stuart and Redenbaugh 1987; Walker *et al.* 1979, Walker and Sato 1981). Leaf petioles (1 cm in length) are cut from greenhouse-grown plants and sterilized in 2.6% sodium hypochlorite for 5 min. The petioles are placed onto SH (Schenk and Hildebrandt 1972) medium containing 25 μM NAA (naphthaleneacetic acid) and 10 μM kinetin (SHNAAK medium) in petri dishes. The petri dishes are incubated at room temperature under fluorescent lights. After three weeks, the newly-grown callus is removed from the petioles, squashed with a spatula, and spread on new SHNAAK medium. Callus maintenance can be continued for six months or more with consistent three-week subcultures without loss of embryo yield or conversion.

To initiate embryos, callus is placed on SH medium plus 50 μM 2,4-D (2,4-dichlorophenoxyacetic acid) and 10 μM kinetin in petri dishes for three days. Afterwards, the callus is placed on SH medium plus 30 mM proline and 12 mM ammonium phosphate in petri dishes. In three weeks, somatic embryos are formed. Embryos are counted for yield measurements, kept on the same petri dishes, and placed at 4°C for 1-4 weeks for further maturation.

To produce plants, embryos are placed in one of two conversion environments: 1) *in vitro* on SH medium, at half strength, with 1.5% w/v maltose substituting for sucrose, or 2) on McCalif mix (peat:vermiculite:perlite, 50:45:5) in trays, without carbohydrates. The McCalif

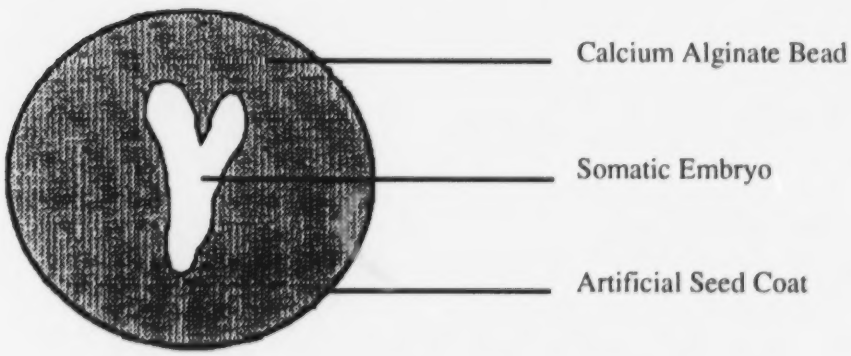
trays are placed inside clear plastic (sweater) boxes with the lid slightly ajar in a growth chamber. For both conversion conditions, a 25°C, 12h/12h light/dark treatment under fluorescent lights is used. In 6 weeks, the number of plants are counted to determine conversion frequency.

In our laboratory, *in vitro* embryo-to-plant conversion frequencies were increased from 0.5% in 1982 to 70% in 1989. Currently, efforts are focused on conducting conversion experiments in McCalif mix in a growth chamber or greenhouse, as well as in the field. Conversion frequencies of 50% in the growth chamber and 20% in the greenhouse have been achieved.

Artificial Seed Production

Somatic embryos can be encapsulated using many hydrogels, but sodium alginate is the most suitable because of ease of encapsulation and lack of damage to the embryos (Redenbaugh *et al.* 1986, 1987a). Making artificial seeds is fairly easy. Using a spatula, an embryo is inserted into a bead of 2% sodium alginate just as the bead drops from a pipet into a complexing bath of 100 mM calcium chloride. After 20-30 min in the bath, calcium alginate beads form. Alfalfa artificial seeds are removed from the complexing solution, washed with water, and planted. See Illustration for drawing of artificial seed and process of making them.

When planted *in vitro* or in a growth chamber, alfalfa artificial seeds can have conversion frequencies equal to that of uncoated embryos. However, when planted in the greenhouse, the frequency is usually less, due to the harsher environment.



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These references are available through interlibrary loan, from university libraries or can be obtained by writing to Dr. Keith Redenbaugh.

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ENZYME EXPRESSION

AND

PRODUCTION

EXPRESSION OF A CLONED GENE IN THE BACTERIUM *ESCHERICHIA COLI*

KEY WORDS: Operon, Lactose, inducer, repressor ß-galactosidase.

BACKGROUND:

Most living organisms, from bacteria to plants and animals, prefer to use the sugar glucose as a carbon and energy source for food. Bacteria only use other sugars when little or no glucose exists in their environment.

The expression of genes that produce the enzymes involved in using other sugars occurs by a process called "induction" or "derepression." For example, the genes involved in lactose utilization are called the *lac* genes, and these genes are expressed only when cellular signals indicate that lactose and not glucose is available (Ptashne and Gilbert, 1970).

The *lacZ* gene, which encodes ß-galactosidase, an enzyme that breaks down lactose, and the *lac* promoter-operator or "control" region (see Figure 1) has been isolated from an *Escherichia coli* bacterium and "cloned" by genetic engineering techniques (Cohen, 1975). The complete *lac* region has been linked to plasmid DNA to generate a molecule that can enter a bacterial cell and replicate within the cell. The cloned *lacZ* gene is activated or "induced" in the presence of either lactose or another molecule that is structurally similar to lactose, called IPTG. During induction, the IPTG causes a "repressor" molecule to be removed from the *lac* control region, allowing the production of ß-galactosidase to begin (see Figure 1). ß-galactosidase activity can be detected when the enzyme attacks a compound called X-gal, which resembles lactose, by the release of a blue color when the X-gal is broken down.

Using modern genetic engineering techniques, genes from a variety of organisms may be linked to plasmid DNA and introduced into bacterial cells. If these genes are joined to the *lacZ* gene control region, their expression may be activated by adding IPTG, or eliminated by removing IPTG. The ability to control gene expression is extremely important in the genetic engineering field (Weinberg, 1985).

STATEMENT OF PROBLEM:

This experiment is designed to compare the activity of the cloned *E. coli lacZ* gene in the presence and absence of the inducer molecule IPTG.

METHODS AND MATERIALS:

Bacterial strains: *E. coli* JM101 - cannot produce ß-galactosidase
E. coli JM101 carrying plasmid pUC12 - cloned *lacZ* gene produces ß-galactosidase (NOTE: The strain containing pUC12 must be grown in the presence of ampicillin to maintain the plasmid)

Media: Sterile LB broth (10 g tryptone, 5 g yeast extract, 10 g sodium chloride per liter water, pH 7.4)
or Sterile LB agar plates (same as above, except 15 g of agar added per liter)

Chemicals: 100 mM IPTG, prepared in distilled water
2% X-gal, prepared in dimethylforamide

Equipment: Sterile 1 ml pipets
Sterile test tubes and racks or sterile petri dishes
Sterile bent glass rods (Method 2)
Shaking incubator at 37°C or incubator chamber at 37°C

Method 1:

1. Prepare 4 sterile tubes containing 5 ml of LB broth per student. Add 0.1 ml of X-gal to each tube, and 0.1 ml IPTG to 2 of the 4 tubes.

2. Aseptically transfer JM101 bacteria to a tube containing X-gal + IPTG and to a tube containing X-gal alone. Do the same for the JM101 bacteria that carry the pUC12 plasmid using the other 2 tubes. Label all 4 tubes carefully.

3. Allow the bacterial cells to grow at 37°C for about 18-24 hours in a shaking incubator.

4. At the end of the incubation, make sure that all tubes show turbidity, i.e., good cell growth, and look to see which tubes contain a blue color. This result is an indicator of ß-galactosidase activity in the bacteria, and this activity should appear only in bacteria that carry a functional *lacZ* gene, and only when the inducer IPTG is added to the bacteria.

Method 2:

1. Add 0.1 ml of X-gal to each of 4 LB agar plates per student and quickly spread the liquid across the surface of the plates with a sterile bent glass rod. (Glass rods may be sterilized by dipping in alcohol, then passing them quickly through a flame, and cooling for a few seconds before use.) Add 0.1 ml of IPTG to 2 of the plates and spread across the plate with a bent glass rod. Label what additions were made to the plates on the bottoms of the petri dishes.

2. Using a sterile pipet, spread JM101 cells on an X-gal plate and on an X-gal + IPTG plate. Repeat for the JM101 cells that carry the pUC12 plasmid on the other two plates. Label the plates with the bacteria that were inoculated on them.

3. Invert the plates and put in the 37°C incubator; allow to grow for 18-24 hours.

4. Look for cell growth on the surface of the plates, and note the presence of any blue color. Expected results are described in step 4 of Method 1.

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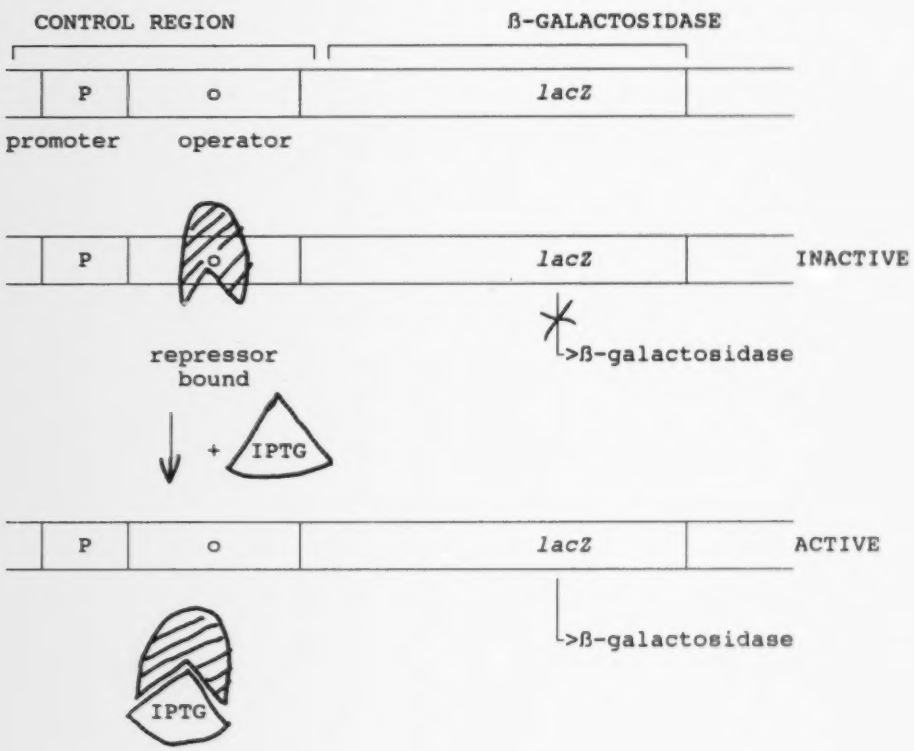
FIGURE 1. THE *LACZ* GENE.



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A LABORATORY FOR DETERMINATION OF THE CONTROL OF GENE EXPRESSION IN THE *LAC* OPERON

KEY WORDS: Operon, Gene Expression, ß-galactosidase, *E. coli.*

BACKGROUND:

The *lac* operon is not only a model system for transcriptional control of gene expression (Beckwith, 1978), but has been a valuable genetic tool in biotechnological applications of recombinant DNA. Regulatory regions of the *lac* operon have been fused to genes from eukaryotic and prokaryotic sources to control their expression and analyze their genetic regulation, or protein structure and function. In addition, regions of the *lac* operon have been an important feature of the most useful plasmid vectors (Reviewed by Silhavy and Beckwith, 1985). Therefore, for students who would like to be involved in recombinant DNA technology, a complete understanding of the structure and regulation of the *lac* operon is essential.

STATEMENT OF PROBLEM:

The purpose of this paper is to present a laboratory designed for the student to determine how positive and negative effector molecules regulate gene expression in the *lac* operon.

INTRODUCTION

The level of expression of the *lac* operon genes can be measured by assaying the activity of Gz, the first structural gene of the *lac* operon. In the cell, Gz hydrolyzes the disaccharide lactose into glucose and galactose. *In vitro*, the activity of Gz can be assayed colorimetrically using the artificial substrate o-nitrophenyl-ß-D-galactopyranoside (ONPG). ONPG is hydrolyzed to a yellow product by the Gz enzyme (Figure 1).

In order for Gz to be expressed in a cell culture, the *lac* repressor, the product of the *lac*I gene, must be inactivated. When lactose or isopropyl-ß-D-thiogalactopyranoside (IPTG) is present in the growth medium, the binding activity of the *lac* repressor is changed and the *lac* repressor does not block transcription of the *lac* operon genes. Transcription of the *lac* operon can be additionally stimulated by the presence of a high intracellular concentration of cyclic 3' 5' adenosine monophosphate (cAMP). cAMP interacts with the catabolite activator protein (CAP) to enhance the binding of RNA polymerase to promotor region of the *lac* operon.

In this laboratory, the activity of Gz will be measured in two different strains of *E. coli*: RA1310 (*lac*I-), a strain which contains a mutant repressor gene so that the *lac* operon is derepressed and Gz is synthesized continuously; and RA1431 (*lac*I+), which synthesizes Gz only after addition of certain compounds such as IPTG or lactose to the growth medium.

METHODS AND MATERIALS:

The bacteria used are K12 derivatives of *Escherichia coli* and include RA1310 (*lac*I-) and RA1431 (*lac*I+). The chemicals necessary for induction of the *lac* operon and the assay of ß-galactosidase (Gz) activity include o-nitro-phenyl ß-D-galactopyranoside and isopropyl ß-D-thiogalactopyranoside. Cell permeablization requires N-lauroylsarcosine. All chemicals can be obtained from Sigma Chemical Co. Other supplies are typically found in most high school science departments. Bacterial strains to complete the laboratory presented below are available on request. An instructors guide for this project may be found in the reagents section of this book.

LABORATORY OBJECTIVES

Student objectives for this laboratory include: 1) Review the control of gene expression in the lac operon; 2) Compare the level of the expression of Gz in *lacI-* and *lacI+* strains of *E. coli*; 3) Measure the effect of inactivating the *lac* repressor with IPTG on the level of expression of Gz; and 4) Measure the effect of activating CAP with cAMP on the level of expression of Gz.

How to Prepare the Lab

In general, this lab is easy to set up but initially it does demand a lot of time in preparing the stock solutions used in preparation of the minimal media. Minimal media is prepared according to Miller (1972) using 1.5% glycerol or glucose as a carbon source. Supplies necessary for minimal media preparations are found in most high school and college science departments.

The bacteria used are K12 derivatives of *Escherichia coli* and include RA1310 (*lacI*$^-$) and RA1100 *lac*I$^+$). I keep these strains frozen at -20°C in 50% glycerol for long term storage from year to year. The week preceding the laboratory, cells are subcultured from the frozen stocks and tested to ensure that the working cultures are free from contaminants. Several hours before the lab, the strains are subcultured fro saturated overnight cultures at 37°C in a shaking water bath. Cells can also be grown without aeration at 37°C in a large bottomed flask but the levels of Gz induction are not as great.

To assay ß-galactosidase (Gz) activity, the artificial substrate, ONPG (o-nitro-phenyl ß-D-galactopyranoside), is used ONPG (1.0 mg/ml) is dissolved without heating in 0.15 M sodium phosphate at pH 7.0. Since the ONPG solution will turn slightly yellow if left at room temperature overnight due to a low rate of spontaneous hydrolysis, I prepare it on the day before the laboratory and refrigerate it until the lab to avoid students questioning the slightly yellow colored substrate. The lactose analog, IPTG (isopropyl ß-D-thiogalactopyranoside), is used to induce gene expression in the lac operon. IPTG is mixed in water in a 100 mM stock solution and can be stored at -20°C. To induce a cell culture, 1 ml of IPTG is added for every 20 mls of cell culture. A 1.0 M stock solution of lactose can also be used to induce gene expression in the lac operon. It has the advantage of being significantly less expensive than IPTG, however, Gz activity increases less rapidly than when IPTG is used for induction.

Solubilization of the cell membrane requires either toluene or chloroform as a nonpolar solvent and either 1% SDS or 5% N-lauroylsarcosine as a detergent. As a result of this treatment, the cells are permeable to the artificial substrate.

Suggestions for Organization of Lab Time

The following outline is a guideline for the amount of time to spend on each portion of the laboratory. In the course where this lab is taught, the laboratory time is limited to 50 minutes.

5-10 min Introduce the lab and explain the experimental design i.e. the cultures, growth conditions and supplies for Gz assay.

Immediately following the brief introduction, 1/2 of flask #3 is poured into #5 and 1/2 of flask #4 is poured into #6. For maximal expression of Gz activity, flasks #5 and #6 should be constantly aerated.

10-30 min Students sample contents of flasks #1-#4 and calculate expected color of tubes.
30-40 min Students sample contents of flasks #5 and #6.

Flasks #5 and #6 are sampled later because at least 20 minutes are required for #5 and #6 to synthesize enough B-Gz to be easily detected during the lab.

40-50 min Review observed vs expected results.

Materials

The list of materials provided below are for a lab of 24 students working in groups of two.

Media

The minimal media is prepared fresh before each lab using M9 broth (7 g Na_2HP_4, 3 g KH_2PO_4, 1 g NH_4Cl per 1 L of water) and several stock solutions mixed as described below.

Solution	Volume (mls)
M9 Broth	400
50% sugar (glucose or glycerol)	12
$MgSo_4$ (1.0 M)	0.40
$FeCl_3$ (10 M)	0.12
$CaCl_2$ (0.10 mM)	0.40
amino acids (10 mg/ml)	1.6
B1 (4.0 mg/ml)	0.40
Biotin	0.40

Before the lab, the media is distributed in 250-ml flasks as described below.

Flask #	Medium	Volume
#1	M9 glucose	30 mls
#2	M9 glycerol	30 mls
#3	M9 glucose	60 mls
#4	M9 glycerol	60 mls
#5	IPTG	0.30 mls
#6	IPTG	0.30 mls

Chemicals

ONPG: 180 mls of 1.0 mg/ml ONPG dissolved in 0.15 M sodium phosphate at pH 7.0.

IPTG: 0.70 mls of 100 mM dissolved in water

Toluene: 12 dropper bottles/lab

Sarcosyl: 5% N-lauroylsarcosine in water distributed in 12 dropper bottles.

Other supplies

6 pi-pumps

2 water baths: One bath contains a plastic test tube rack with 72 spaces for incubating Gz assay tubes. The other needs an apparatus for aerating the cultures. I use an aquarium pump linked to an aquarium air manifold with silicone tubing leading to the flasks to provide

the aeration.

7 2-ml pipets that are placed in each flask at the beginning of the experiment.

72 13 x 100 mm disposable test tubes

12 test tube racks

12 wax pencils

PROCEDURE

Students work in groups of 2 or 3. Each group measures the Gz activity found in six cultures (Table 1). Use the flow diagram given in Figure 2 to test for the presence of Gz in the different cell cultures. At the beginning of the lab, cultures 3 and 4 will be split. One half of the cultures will be added to flasks 5 and 6, respectively, which each contain IPTG to inactivate the *lac* repressor. Wait approximately 30 min after the cultures are split before measuring the Gz activity in flasks 5 and 6. Using your knowledge of gene regulation in the *lac* operon, fill in the repressor activity and CAP activity columns in Table 1. Place a (+) in the column if you expect the protein to be active, and a (-) if you expect the protein to be inactivated due to mutation or due to physiological conditions which exist in the cell as a result of its growth medium.

Based on the predicted activity of the *lac* repressor and the CAP, fill in the amount of yellow color that you would expect to be present after the cultures were assayed for Gz activity. At the conclusion of the lab, compare your expected result with your observed results. Discuss any deviations from your expectations with your instructor.

Table 1. ß-galactosidase activity under various conditions of growth.

Flask No.	Culture	Relative (cAMP)	Repressor Activity	Cap Activity	Yellow Expect.	Color Obs.
1.	RA1310 (*lacI*-)	low				
2.	RA1310 (*lacI*-)	high				
3.	RA1431 (*lacI*+)	low				
4.	RA1431 (*lacI*+)	high				
5.	RA1431 (*lacI*+)	low in IPTG				
6.	RA1431 (*lacI*+)	high in IPTG				

Figure 1. Hydrolysis of ONPG by ß-galactosidase (Gz).

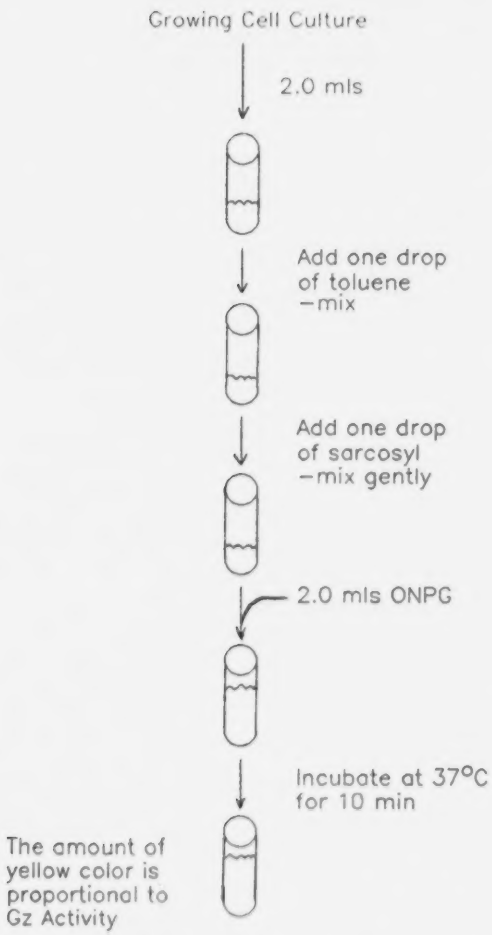
Growing Cell Culture
2.0 mls
Add one drop of toluene –mix
Add one drop of sarcosyl –mix gently
2.0 mls ONPG
Incubate at 37°C for 10 min
The amount of yellow color is proportional to Gz Activity

Figure 2. Flow Chart for Gz Assay

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THE DETECTION OF BETA-GLUCURONIDASE ENZYME ACTIVITY AS A METHOD FOR IDENTIFYING *ESCHERICHIA COLI*

KEY WORDS: ß-Glucuronidase, *Escherichia coli*, Serial dilution.

BACKGROUND:

Because production of the ß-glucuronidase enzyme is largely limited to *E. coli* and several closely related but less numerous bacteria, it can be used as a relatively specific marker to identify *E. coli* in the presence of many unrelated bacteria. *E. coli* is a member of a family of intestinal (enteric) bacteria, the *Enterobacteriaceae*, which inhabit the intestinal tracts of humans and other warm-blooded animals. In addition to *E. coli*, several well-known disease producing bacteria (pathogens) also belong to this family including *Salmonella*, the causative agent of *Salmonella* food poisoning and typhoid fever. The presence of *E. coli* in foods is used as a universal indicator of possible fecal contamination. It is important, therefore, for the food industry to have a rapid and accurate method for identifying this bacterium in various edible products.

The standard method used to identify *E. coli* takes 5-7 days and involves the sequential inoculation and incubation of several types of media. This identification period can be shortened to 24 hours or less, however, by counting bacteria that produce ß-glucuronidase. Enzyme activity is detected by adding compounds to the bacterial growth media which are cleaved or split specifically by bacteria producing the enzyme. Two such substances used for this purpose include a compound called MUG which produces a fluorescent substance when cleaved, and the compound used in this exercise abbreviated as BCIG (or X-GLUC) (figure 1) which produces an intense blue color when split by the enzyme.

STATEMENT OF PROBLEM:

To detect ß-glucuronidase positive bacterial colonies on the surface of agar plates as a method of identifying *E. coli* in a food sample within 24 hours.

METHODS AND MATERIALS:

Materials:

Flask (2000 ml) for agar preparation
Raw hamburger meat or raw pork sausage
Overnight culture of *E. coli*
Sterile water (90 ml in wide mouth bottle)
Sterile pipets (1 ml)
Pipeting Bulb
Agar plates* (see resource section)
Sterile Water Dilution blanks (9 ml and 99 ml - capped 18 x 150 mm test tubes and 8 oz. capped medicine bottles)
Alcohol
Bunsen burner
Glass spreaders (bent glass rods)
Sterile tongue depressors
Balance
Felt Marking Pen or Wax Pencil

1. Using a sterile tongue depressor, weigh 10 g of meat into 90 ml of sterile distilled water. Replace the cap on the bottle and shake vertically 20 times in a 1 ft arc. This preparation is a 1/10 (10^{-1}) dilution of the meat.

2. After settling, use a sterile pipet with pipeting bulb attached to transfer 1 ml from the above dilution to a 9 ml sterile dilution blank producing a 1/100 (10^{-2}) dilution. Mixing is accomplished by holding the tube firmly between the thumb and index finger and with the index finger of the other, striking the lower side of the tube creating a swirling motion.

3. From the overnight *E. coli* culture (positive control), transfer 1 ml from the culture tube to a 99 ml dilution blank producing a 1/100 (10^{-2}) dilution and mix with a swirling action as above. With a new pipet, transfer 1 ml from the 1/100 (10^{-2}) dilution to a 99 ml dilution blank producing a 1/10,000 (10^{-4}) dilution. Mix as described above. Finally, with a new pipet, transfer 1 ml from the 1/10,000 (10^{-4}) dilution to 99 ml blank producing a 1/1,000,000 (10^{-6}) dilution. Mix by swirling action and label all dilutions appropriately (figure 2).

4. Pipet 0.5 ml from each of the meat dilutions (10^{-1} and 10^{-2}) onto the surfaces of separate agar plates. With a felt pen, label the dilution level on the cover of each plate. Spread the inoculum evenly and thoroughly over the entire surface of the agar with separate glass spreading rods sterilized by brief flaming after being dipped in alcohol. Repeat the procedure for the *E. coli* culture using only 0.1 ml of the 10^{-6} dilution. After 30 min, invert the plates and copy the dilution level onto the bottoms of the plates. Incubate the inverted plates overnight at 37°C or at room temperature if there is no incubator. Observe the plates every hour or two from about 16 hours on for those growing at 37°C (it will take longer for the room temperature plates).

5. Record the number of blue colonies on each plate. To calculate the original density of presumptive *E. coli*, multiply the number of blue colonies counted on each plate by the inverse of the dilution, i.e. 1/100 [10^{-2}] = 100/1 [10^{2}] etc. Then multiply by 2 to convert the 0.5 ml plated to bacteria per g of meat and by 10 to convert the 0.1 ml plated to bacteria per ml. Record your results as the number of presumptive *E. coli* per gram or per ml.

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RESOURCES:

Disposable Petri Dishes - (100 X 15 mm) - 60 dishes from Carolina Biological Supply Co. Burlington, NC. 27215 (800-334-5551) Cat. No. 74-1350. *Agar Medium - Nutrient Agar - 1/4 lb. - from above Cat. No. 78-5300. Prepare according to directions on label. Add BCIG as directed below. Prepare plates at least 1 day before running the experiment.

*Tergitol 7 Detergent (to inhibit gram-positive bacteria) 100 ml from Sigma Chemical Co. P.O. Box 14508, St. Louis, MO 63178 (800-325-3010) Add 0.1 ml per l of nutrient agar before sterilizing

*BCIG - sodium salt (Request 100 mg sample) - from Biosynth International, Inc. P.O. Box 541, Skokie, Il 60076 (312-674-5160) --(Add either 50 or 100 mg to 1 L of sterilized nutrient agar cooled to about 50°C and mix by rotating the flask just before pouring the plates -- 1 L prepares about 50 agar plates)

Nutrient Broth - 10 tubes - from above Co. Cat No. 82-6120

E. coli - tube culture - from above Cat. No. 15-5065

Pipet-Pump (Blue Pi-Pump) from above Cat. No. 73-6870

Disposable polystyrene pipets, serological, sterile (1 x .01 ml) shelf pack (50) from above Cat. No. 73-6045
Flask, Erlenmeyer, measuring - 2000 ml from above Cat. No. 72-6702

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Figure 1

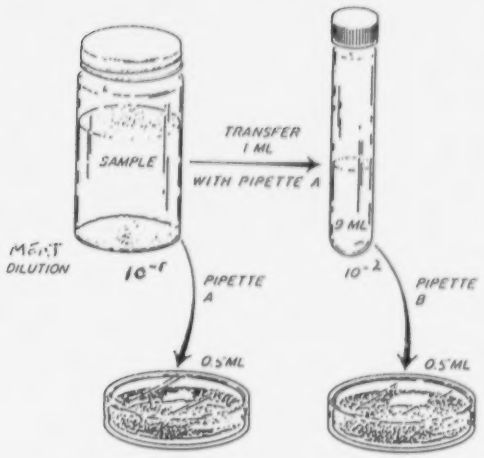

Figure 15-1 Diluting the sample for the spread plate, a quantitative plating method.

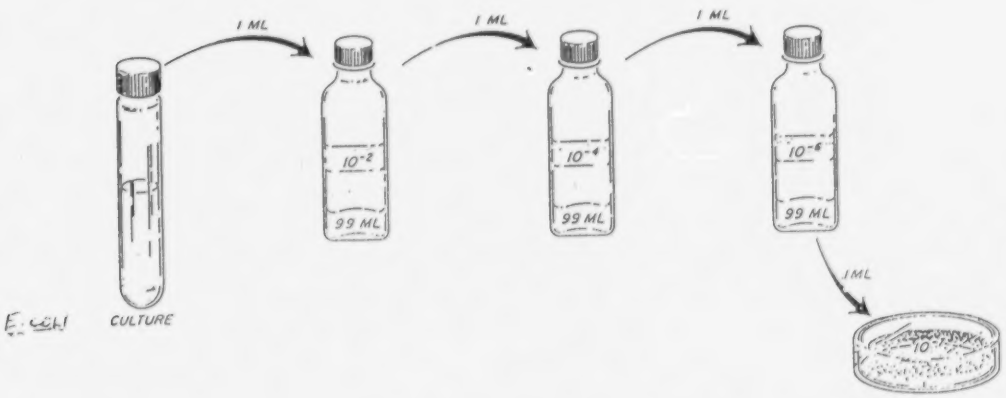

Figure 17-1 Dilution procedure

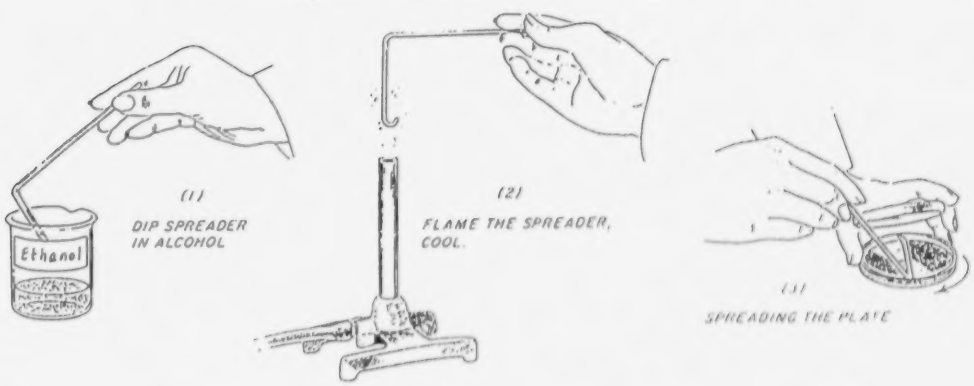

Figure 2

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THERMOSTABLE AMYLASE FROM RECOMBINANT *ESCHERICHIA COLI*

KEY WORDS: Amylase, Plasmid, Repression.

BACKGROUND:

Escherichia coli is one of the best understood bacteria from the genetics standpoint, making it attractive for production of recombinant proteins of value in biotechnology. Like many other Gram-negative bacteria, it suffers from the disadvantage of retaining the product within the cell, leading to need for cell disruption and expensive purification to recover the product. The recombinant strain you will use has overcome this problem with genetic engineering. The strain carries a plasmid which contains a 6 kb DNA fragment from the thermophilic bacterium *Bacillus stearothermophilus* on a multicopy plasmid pBR322. This fragment codes for a thermostable amylase which is secreted extracellularly for ready recovery from the broth following cell removal. This enzyme is utilized for industrial conversion of starch to oligomers of glucose, which are in turn used for production of sweeteners, fuels, and other products.

STATEMENT OF PROBLEM:

The purpose of this experiment is to determine whether production of this amylase enzyme is sensitive to glucose in the medium (catabolite repression) which would preclude use of some carbon sources. Selection of the appropriate growth conditions may be as important in successful production of genetically engineered products as the recombinant DNA techniques used for organism modification.

METHODS AND MATERIALS:

Place 5 ml of *E. coli* EC147 in 100 ml fresh L broth with and without 1% glucose and shake in a 250 ml flask at 37°C until turbidity is observed to be increasing. Take 2 ml samples and first measure the turbidity at 550 nm as a measure of the number of bacteria. Then remove the bacteria by filtration using a .22 or .45 μ filter or use of a microcentrifuge (1300 g for 30 seconds) and save the supernatant on ice for amylase assay. Compare the growth and specific amylase production (amylase units/turbidity units) with time through the period of exponential growth and determine if glucose inhibits production.

AMYLASE ASSAY-STARCH IODINE METHOD

REAGENTS:

0.2M acetate buffer (6.56 g NaAc (sodium acetate) + 1.2 g/HAc (acetic acid), adjust to 500 ml with H_2O, adjust pH to 5.25 with NaOH)
0.5M $CaCl_2$
1% starch (Difco soluble starch) solution in H_2O
1N HCL
3% KI/0.3% I_2

REACTION COCKTAIL:

5 ml 0.2M NaAc
1 ml $CaCl_2$
1 ml starch solution
3 ml dd (deionized distilled) H_2O

REACTION:

1. To 0.8 ml of reaction cocktail, add enzyme solution (about 25 μl of a 1.5 unit/ml solution gives a reasonable change in optical density in 5 min).

2. Stop reaction at desired time by immersing tube in ice, add 1 ml of 1N HC1.

3. Add 200 μl of 3% KI/0.3% I_2 solution for color reaction.

4. After tubes have warmed to room temp. (about 15 min) read on spectrophotometer at 620 nm (iodine binding is temperature dependent).

Zero time points should be made by adding enzyme directly before iodine solution with no exposure to assay temperature. Blanks are made by substituting dd H_2O for enzyme solutions. Blanks containing water instead of enzyme should have an optical density of between 1.0 to 1.3.

Standard assay temperature for *B. stearothermophilus* amylase is 70°C. One unit of activity is defined as the amount of enzyme required to release 1 mM of maltose/minute. We assume that all of the starch can be quantitatively converted into maltose. Thus, the subsequent loss of optical density is directly proportional to the amount of maltose released, which in turn is directly proportional to the enzyme activity. The assay is significantly non-linear with time and enzyme concentration. One should set up the assay such that replicates of several time points are used, and that changes in optical density between times points are small. Beware of the source of the starch; there are great differences in iodine binding capacity with different suppliers.

REFERENCES:

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Note to instructor: Strong catabolite repression should be observed. EC147 should be retained on L slants with 50 mg/ml ampicillin. No antibiotic need be added during experimentation. BLI containment should be used (see summary of NIH Guidelines).

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THE SCREENING OF BACTERIA FOR CELLULASE ACTIVITY

KEY WORDS: Cellulase, Endoglucanase.

BACKGROUND:

Cellulose is the most abundant carbohydrate polymer in nature, and together with lignin and hemicellulose, form the major constituents of higher plants. Microorganisms able to break down cellulose have great economic potential for pollution abatement, for increasing the utilization of renewable plant biomass as animal feed, and for the production of fuels and chemicals by fermentation (figure 1).

The degradation of cellulose occurs due to the activities of a multienzyme complex termed cellulase. The cellulase complex consists of three components: exo-glucanase, endo-glucanase and cellobiase.

The ability to produce endoglucanase can be tested for by growing bacteria on carboxymethyl-cellulose (a soluble form of cellulose) agar medium and flooding with Congo red. The use of Congo red as an indicator for cellulose degradation provides the basis for a rapid and sensitive screening test for cellulolytic bacteria. Where the cellulose has been degraded, there will be a clear yellow zone around colonies of bacteria exhibiting endoglucanase activity. Where intact molecules of cellulose are left, there will be a reaction with the Congo red solution giving a red color.

STATEMENT OF PROBLEM:

To test for cellulase activity in bacteria.

MATERIALS:

Bacterial strains: Broth cultures (LB or nutrient broth) of *Escherichia coli* C600, *Bacillus subtilis*, and *Cellulomonas fimi* 484 (all are obtainable from the American Type Culture Collection).

Media: LB agar medium modified by the inclusion of carboxymethyl-cellulose (0.1% w/v, sodium salt, low viscosity grade, Sigma Chemical Company) (see appendix).

Chemicals: Congo red (1.0 mg/ml, Sigma Chemical Company), NaCl (1.0 M solution, Sigma Chemical Company).

Equipment: Standard incubator, platform shaker.

METHODS:

1. Divide a carboxymethyl-cellulose agar plate into 3 sectors, and label 1, 2, and 3.
2. Inoculate each sector of the plate with a drop of the broth cultures provided: 1. *E. coli*, 2. *B. subtilis*, and 3. *C. fimi*.
3. Incubate the plate (in inverted position) overnight (10-12 hr) at 37°C.
4. Flood the plate with Congo red solution and shake for 15 min at room temperature.
5. Discard the Congo red solution and rinse with 1.0 M NaCl for 15 min at room temperature.

6. Discard the NaCl wash, and observe the plate for discoloration.

Additional Activity

Bacteria could be isolated from soil and these could be screened for amylase activity.

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CONCEPT OF CELLULOSE RECOVERY AND UTILIZATION TO PRODUCE ANIMAL FEED, FUELS AND CHEMICALS.

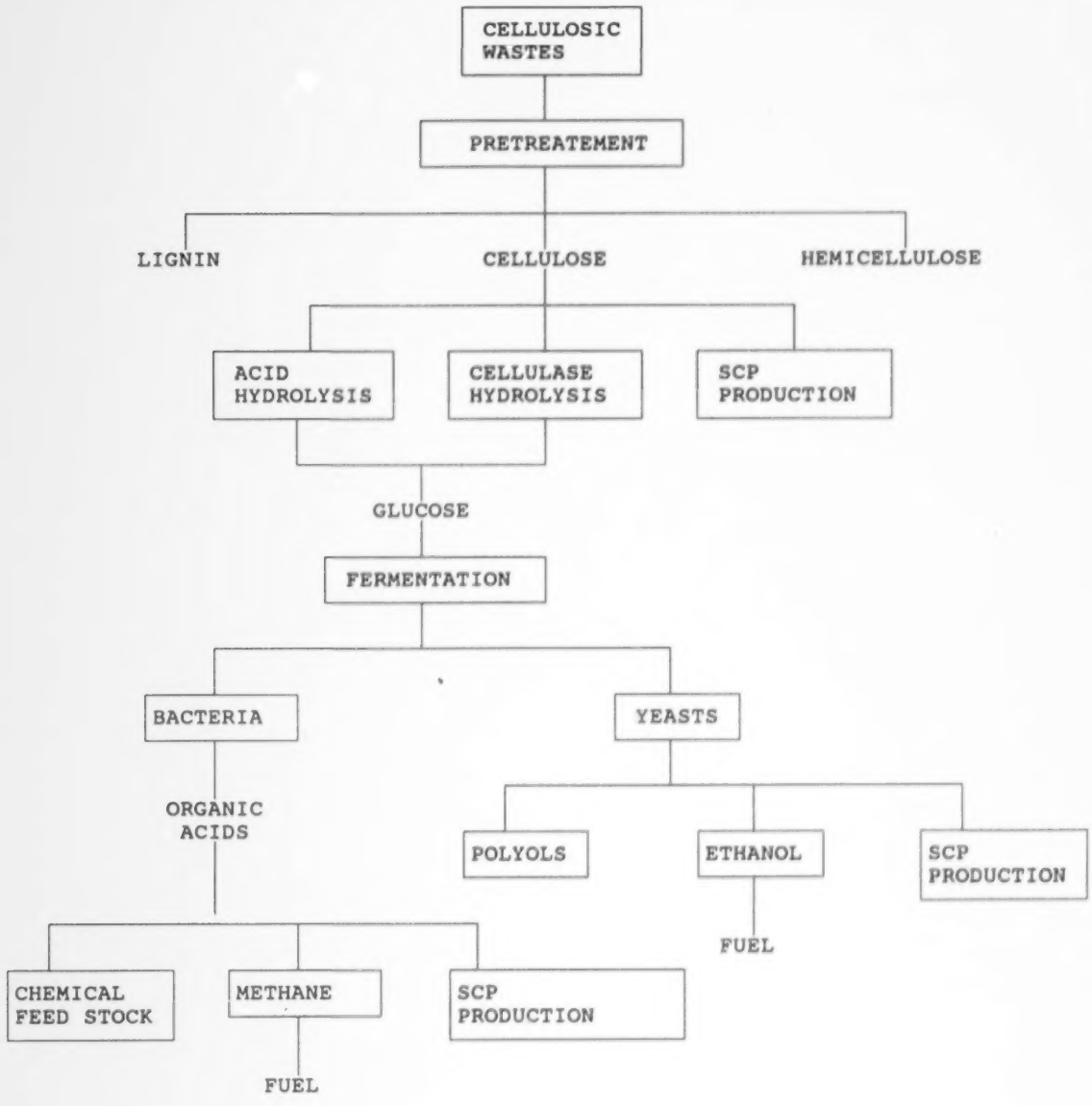

Figure 1

DETERMINATION OF THE AMYLOLYTIC ACTIVITY OF RUMINAL OR OTHER BACTERIA

KEY WORDS: Rumen, Starch digestion, Amylase.

BACKGROUND:

Ecological studies of ruminal microbiology have attempted to identify which species of microorganisms are most numerous in the rumen under a particular dietary regime. One method employed uses selective media to determine the numbers of organisms performing a particular metabolic activity such as cellulose or pectin digestion. Another approach is to characterize isolates obtained with general purpose, non-selective media, determine the most numerous organisms, and what metabolic abilities they exhibit. In both these approaches it is implied that the predominant species (most numerous) are the organisms most important in the digestion of the particular dietary component in question. Clearly this is an important consideration in the determination of the relative importance of a particular organism to the function in question. These approaches, however, underestimate or ignore the capacity of individual species to perform a given metabolic function. A more complete ecological analysis also includes measurements of the levels, characteristics, and regulation of the enzyme activities required to perform the metabolic function of interest (i.e. amylases for starch degradation). In addition, such information is valuable in devising strategies for the genetic manipulation of species to be reintroduced into ruminant livestock or identification of species amenable to exploitation in commercial processes.

STATEMENT OF PROBLEM:

The objectives of the current study are to determine the relative amylolytic activities of starch-degrading ruminal bacterial species, to identify the products of amylolytic attack, and to examine the effects of culture conditions on enzyme production.

METHODS AND MATERIALS:

Organisms and growth conditions. Cultures of ruminal bacteria are grown in a complex medium containing 0.2% glucose, maltose, or starch as the carbon and energy source. [Since ruminal bacteria are difficult to cultivate and require some specialized equipment. I would recommend the use of organisms that can be cultivated under aerotolerant conditions, for instance; *Bacillus subtilis*]. Samples are collected by centrifugation of cultures (10,000 X g, 20 min, 4°C) and cells are resuspended in 100 mM potassium phosphate buffer, pH 6.8. Cells and cell-free supernatant fluid samples are analyzed immediately for amylase activities.

Amylase assay. Amylase activity of samples is determined by monitoring the increase in reducing sugar formation from starch using the dinitrosalicylic acid reagent.

Reagents:

1. Dinitrosalicylic acid reagent:
   10 g 3,5 dinitrosalicylic acid (DNS)
   2 g phenol (crystals)
   0.5 g sodium sulfite
   200 g sodium potassium tartrate in 1 L 1% sodium hydroxide
2. Starch substrate: 1%
   1 g soluble potato starch
   dissolve by heating (boiling water bath) in 100 ml 100 mM potassium phosphate (pH 6.8)

Assay:
1. Combine;
   0.5 ml substrate
   0.5 ml appropriately diluted enzyme sample
2. Incubate;
   39°C, 10 min
3. Stop;
   add 1.5 ml DNS reagent
4. Mix;
   heat 100°C, 15 min
5. Cool
6. Read at 640 nm
   Against glucose standard (0-1000 μg/ml)

Controls:
1. 0.5 ml substrate
   1.5 ml DNS reagent
   then add, 0.5 ml enzyme solution
   Mix, boil, etc. as above.

Reducing sugars formed are estimated by comparison to a glucose standard. One enzyme unit equals one μmol of glucose equivalent formed per minute.

Products of amylose digestion. The products of amylolytic attack are determined by measuring the release of oligosaccharides from amylose. Aliqouts (10 ml) of cell-free culture fluid from cultures grown overnight in maltose-containing medium are combined with 10 ml of an amylose solution (2% in 50 mM potassium phosphate buffer, pH 6.8). These mixtures are incubated at 39°C and 1 ml samples are removed at various times (0-24 h), and desalted with a mixed bed ion exchange resin (90 mg/ml, Bio-Rad AG 501-X8D). The oligosaccharides in the samples (20 μl) are separated by high-pressure liquid chromatography on a Regis reversible amino column (Regis Chemical Co., Morton Grove, IL, 250 mm X 4.6 mm), with 70% acetonitrile as the eluant (2 ml/min). Peaks detected by refractive index (Waters model 401 differential refractometer) are processed with a Hewlett-Packard model 3392a integrator and identified by comparison to retention times of authentic standards. This method, while having the advantage of being quantitative, requires expensive reagents and specialized equipment.

Alternatively, the products of amylose digestion can be determined qualitatively using a thin layer chromatography method that is much less expensive and requires little specialized apparatus.

Solvent:

Nitroethane: Ethanol: Water, 1:3:1, approximately 100 ml in a chromatography tank. Best if made fresh daily.

Detection Spray:

Dissolve 200 mg N-(1-Naphthyl) ethylene-diamine hydrochloride in 97 ml methanol then add 3 ml concentrated sulfuric acid.

Chromatography plates:

Whatman 150A K5 Silica Gel, 250 micron layer thickness, 20 X 20 cm.

Samples:

Standards: 10 mg/ml of each oligosaccharide in water. Apply 1 μl.
Samples: Same as those describe for HPLC method. Desalting not as important in this method and can be deleted if small samples are used. Apply 10 μl.

Methods

1. Add solvent to chromatography tank at least 30 min prior to use
2. Apply samples or standards 1-2 μl at a time approximately 1/2 inch apart and 1/2 inch from bottom of plate (should be above level of solvent when placed in tank). If multiple applications are required (frequently the case with samples) it is helpful to dry spots with a hair dryer to minimize spreading.
3. Place chromatography plate in tank. Do two ascents to resolve oligosaccharides well, drying plate between ascents. You can use a hair dryer to speed up drying.
4. After final ascent, dry plate and spray with detection spray. Saturate plate well with spray. Place in drying oven at 100°C for 10 min to visualize spots. If spots are light you can spray again to increase their intensity.

Chemicals. For these experiments, a preparation of soluble potato starch (Sigma Chemical Company, St. Louis, MO, cat. no. S-2630) that contained no detectable glucose, maltose or other oligosaccharides (as determined by HPLC method) is used as both the growth and amylase assay substrates (this is relatively important as many "soluble" starch preparations contain significant amounts of soluble sugars). Oligosaccharide standards, amylose, and other chemicals can be obtained from Sigma Chemical Co., St. Louis, MO.

REFERENCES AND RESOURCES:

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ENZYME PRODUCTION BY CELLULOSE-DEGRADING BACTERIA

KEY WORDS: Cellulase, Thermophilic, *Thermomonospora*, Endoglucanase.

BACKGROUND:

Cellulose, the world's most abundant organic material, is degraded slowly in nature. Most of the carbon trapped in cellulose is recycled by microbial activity (Berquist *et* al, 1987). Therefore, it is important to understand how these microbes perform this process. The cellulose-degrading enzymes (cellulases) secreted by cellulolytic microbes are diverse and complex, both in their physical properties and in their catalytic activities. The cellulose polymer is composed of beta-1,4-linked glucose molecules. To break down this polymer (which might consist of several thousand glucose residues), special cellulases called endoglucanases must first break internal polymer bonds to yield free chain ends. These polymer fragments are then broken down by exoglucanases into two-glucose units called cellobiose. Cellobiose is a free sugar which can then be taken up by the microbial cells. Once inside, cellobiose is broken down to two glucose molecules which can then serve as a carbon and energy source for continued microbial growth (Bernfield, 1955).

During the last decade, the cellulases of thermophilic (heat-loving) microbes have attracted much attention because they have great potential in the biotechnology of fuel generation from agricultural and municipal waste (Eriksson, 1979). If these cellulases can quickly and efficiently turn waste cellulose into soluble sugars which can then be fermented to fuel alcohols, the two problems of waste disposal and fuel availability could be considerably decreased. On an industrial scale, the thermophilic cellulases are more rugged and durable than those produced by microbes growing at lower temperatures and they operate at temperatures which require less cooling of cultures; some even operate best above pasteurization temperature (62°C) which prevents the growth of undesirable microbes in the fuel generation system (Ljungdahl, and Eriksson, 1985). However, to make maximum use of these cellulases in any biotechnological application, we must understand the mechanisms which regulate their biosynthesis within the microbial cell (So that we can get the highest possible enzyme yield) and the characteristics of each individual enzyme (so that we know what to expect from its activity when we want them to do specific tasks).

In this experiment, a thermophilic bacterium, *Thermomonospora curvata*, will be grown on cellulose as the sole carbon and energy source at 55°C (over 130°F). This bacterium grows in long filaments (like some fungi) which attach themselves to the cellulose fiber so that they can constantly stay next to their food supply (see Figure 1 as an example). As it grows, it secretes cellulases from its cells which then attack the cellulose surface and provide it with usable sugars.

STATEMENT OF PROBLEM:

The goals of this experiment are two-fold: 1) to measure the increase in the cellulase activity of culture fluid as *T. curvata* grows on the cellulose; and (2) to determine whether we can detect changes in the pattern of cellulases as the culture matures (patterns change because genes coding for cellulases are often switched on and off in different culture phases, and the cellulases themselves can be changed by other enzymes called proteases).

METHODS AND MATERIALS:

Culture medium.

T. curvata is grown in the following chemically defined medium: 0.05 molar phosphate buffer (pH 8.2), 0.05 molar HEPES buffer (pH 8.2), 0.1% ammonium sulfate, 0.01% magnesium chloride hexahydrate and 0.8% ground absorbent cotton (Johnson and Johnson

surgical grade). The medium is autoclaved in 100 ml aliquots contained in 250 ml Bellco long-necked Erlenmeyer flasks with metal caps. After cooling, aseptically add 0.1 ml of 10% sterile Bacto-yeast extract (Difco type B127) to each flask. The likewise addition of 0.1 ml of 1.0 molar sterile calcium chloride to each flask increases growth and cellulase production. All buffers and other chemicals not source-designated may be obtained from Sigma Chemical Co., St. Louis, MO.

Inoculation and growth conditions.

T. curvata stock cultures may be obtained from American Type Culture Collection. Stock cultures can be preserved indefinitely by freezing aliquots of cultures grown in the above medium. Each flask should be aseptically inoculated with 1 ml of thawed stock culture. Most rapid growth is obtained at 53°-55°C in a shaking incubator. Growth can be obtained in unshaken flasks at temperatures of 45°-50°C, but is somewhat slower and more erratic.

Culture sampling.

Five to 10 ml culture fluid samples should be aseptically withdrawn from flasks at 0,2,4,7 and 10 days of growth (starting cultures on Monday mornings allow samples to be taken during regular school hours). Sterile pipets with broken tips should be used as the cotton fibers in the medium will clog a regular pipet. Culture samples can be clarified by a brief centrifugation or by filtering through a Gelman filter (type DM, 0.8 micrometer pore size). Filter paper should not be used for this purpose, as it traps most of the cellulase and protein due to adsorption. A simple prefiltration apparatus can be made from a 50 ml syringe with a circular piece of small mesh screen which will remove the large particulate matter from the culture sample. All assays can be done on the clarified fluid which contains both extracellular protein and a variety of cellulases.

Assays.

Growth of the organism cannot be determined by spectrophotometric or gravimetric methods, since the cotton fibers are insoluble and cannot be separated from the cells. However, protein accumulation in the culture fluid is proportional to cell mass, and can be used as an estimate of growth. Protein concentrations of culture fluid can be easily obtained within 10 min using the Bio-Rad standard protein assay (the technical bulletin 8L-0275 supplied with the reagent gives complete instructions for both standard and micro assay methods).

Cellulase enzyme content of culture fluid is measured using carboxymethyl cellulose (CMC) as a substrate. CMC is a soluble cellulose derivative which is degraded to sugars by the action of the cellulases in the clarified culture sample. It is a good substrate for specifically measuring the endoglucanases which cleave the molecule at many places along its length. The stock CMC solution is prepared by slowly adding 2.0 g of Hercules type 7L1 CMC to a warm, stirred solution of 0.1 molar MES buffer, pH 6.2. Put 1.9 ml of this viscous solution into test tubes (size 16 x 125 mm), one for each culture fluid sample to be assayed. Add 0.1 ml of culture fluid to a substrate tube to start the enzyme-substrate reaction, mix vigorously and rapidly, then withdraw 0.5 ml and add to 1.0 ml reducing sugar reagent (RSR). [The RSR should be made at least 24 hours in advance according to Bernfeld (5): 1) dissolve 10 g of dinitrosalicylic acid in 200 ml of 2.0 normal NaOH, 2) bring the volume up to about 700 ml with distilled water, 3) warm the solution and stir in 300 g of Rochelle salt (sodium potassium tartrate), 4) after all is dissolved, add water to a final volume of 1 L, then store in a brown bottle in a dark place.] RSR will stop the cellulase reaction and will turn color when boiled with any sample containing reducing sugar such as glucose or cellobiose. Place the tube with the remainder of the reaction mixture into a water bath at 65°C for 10 min to allow the reaction to proceed. Then take another 0.5 ml sample from the tube and add it to another ml of RSR. In this manner, you will have two RSR tubes for each assay, one with a zero time

sample, and one after a 10 min reaction. To determine how much sugar has been released from the CMC by the action of the cellulases, boil all the RSR tubes for 10 min (be sure you have all tubes marked clearly as to sample number and whether they are zero time or reaction tubes), then add 1.5 ml of distilled water to each to give a final volume of 3 ml in each tube. If there is cellulase activity in the culture fluid sample, the RSR tube containing the reaction sample will be darker than its corresponding zero time tube because there will be more reducing sugar liberated from the substrate. To determine just how much is liberated, you must create a standard curve for comparison using a known sugar (glucose is the easiest to use). You can make a simple glucose standard curve as follows: put one ml of RSR in each of four tubes. Add a ml of water in the first, 0.75 ml of water in the second, 0.5 ml in the third and no water in the fourth. Make a glucose solution at a concentration of 0.2 mg per ml. Do not add any glucose solution to the first tube (that is your blank or control tube which gives you a color comparison). To the second tube, add 0.25 ml glucose solution, 0.5 ml to the third and one ml to the fourth. From #1 to #4, the glucose concentrations will be 0, 50, 100 and 200 ug per ml of sample volume. These tubes should be boiled just like the sample tubes. *The final volumes of all tubes after boiling must be 3 ml.*

For the greatest precision in establishing the standard curve and activities of each cellulase sample, the optical density of each tube should be determined using a spectrophotometer set at a wavelength of 540 nm. The optical density (OD) range of the glucose standard curve will be 0-0.4 OD units (see Fig. 2 for example of standard curve). If this instrument is not available, you can still get an estimate by lining up the glucose standard curve tubes, #1 through #4, and placing each sample tube to its closest match along this series. It is obvious that the more tubes you have in your standard curve with this estimation system, the more accurate your estimation system can be. Also, remember that when you calculate the glucose generated in your sample, you must subtract the glucose value in your zero time sample. For example, if the zero time sample is as dark as the glucose standard tube having 50 μg/ml, and the 10 min sample is estimated to have 200 μg/ml, then the sugar generated during the 10 min cellulase reaction is equivalent to 150 μg glucose.

Calculation of cellulase activity.

After the glucose equivalents have been estimated for all samples, the values must be converted into enzyme units. A unit is defined as the production of one micromole of product per min. A micromole of glucose equivalents is 180 ug. Therefore, we can calculate our enzyme activity in units by the following formulae:

$$\frac{\text{ug glucose produced}}{180} = \text{micromoles of product}$$

$$\frac{\text{micromoles of product}}{\text{min of reaction}} = \text{units of enzyme activity}$$

For example, in our reaction system just described, we incubated for 10 min and took 0.5 ml samples. If there were 200 μg of glucose equivalents in that 0.5 ml sample, then total product formed in the 2.0 ml reaction system would be 200 x 4 = 800, (800/180) = 4.44 micromoles; 4.44 micromoles/10 min = 0.444 units. Remember that we only used 0.1 ml of culture fluid, so the cellulase activity of the culture fluid is 4.44 units per ml. We can also calculate "specific cellulase activity" (cellulase units per mg of extracellular protein) by simply dividing the cellulase units per ml by the mg of protein per ml.

If you have prepared the medium correctly, carefully taken samples at the suggested intervals, and quantified protein and cellulase accurately, you will see that the levels of protein and enzyme in the culture fluid increase during the first few days of incubation, then level off and decline toward the end of the experiment in a manner similar to Fig. 3. If facilities are available for the separation of the various enzymes in the mixture, you will also be able to see

that the patterns of extracellular cellulases change as the culture ages. One way of doing that will now be described.

Cellulase concentration and fractionation.

All the extracellular proteins in *T. curvata* culture fluids can be rapidly concentrated in an Amicon Centricon-10 microconcentrator. For comparison of the cellulase and protein profiles, a good contrast can be made between those from early cultures (taken at two or three days growth) and late cultures (taken at seven to 10 days growth). Two ml of each culture fluid is put into the upper reservoir of a microconcentrator. The units are then placed in a small angle-head tabletop centrifuge and spun for several minutes at a maximal speed of 5000xg. The fluid containing the small molecular weight components passes through the filter, while the macromolecules, including the proteins are retained and concentrated. In this manner, a 20-fold concentration of the cellulases can be attained in a short time with little loss of activity. Since the early culture sample will contain less protein and cellulase, it should be concentrated 30-40 fold. This can be easily done by adding more culture fluid to the retentate after centrifuging and repeating the process.

HPLC fractionation of concentrated cellulases.

The cellulases of *T. curvata* and closely related species have been purified by a variety of techniques (6,7). However, fractionation by high pressure liquid chromatography (HPLC) is most rapid and requires only a small amount of sample (usually less than 0.5 ml). A 7.5 mm by 75 mm diethylaminoethyl (DEAE) column (Bio-Rad type 5PW) gives good separation of the cellulase enzyme forms. The column is equilibrated with 0.1 molar phosphate buffer, pH 7.0. After injection of the sample, the cellulases are eluted from the column by a linear 0-0.5 molar NaCl gradient at a flow rate of 0.5 ml per minute. The taking of consecutive fractions at one minute intervals will provide sufficient samples to establish differences in fractionation patterns between early and late cultures. Determine the cellulase and protein content of each fraction and graph those values versus fraction number (or ml of fluid from the column). You will see that the patterns made by samples from early cultures (only two or three days old) will be different than those from older (7-10 day) cultures. There at least five different cellulase enzymes bt *T. Curvata*

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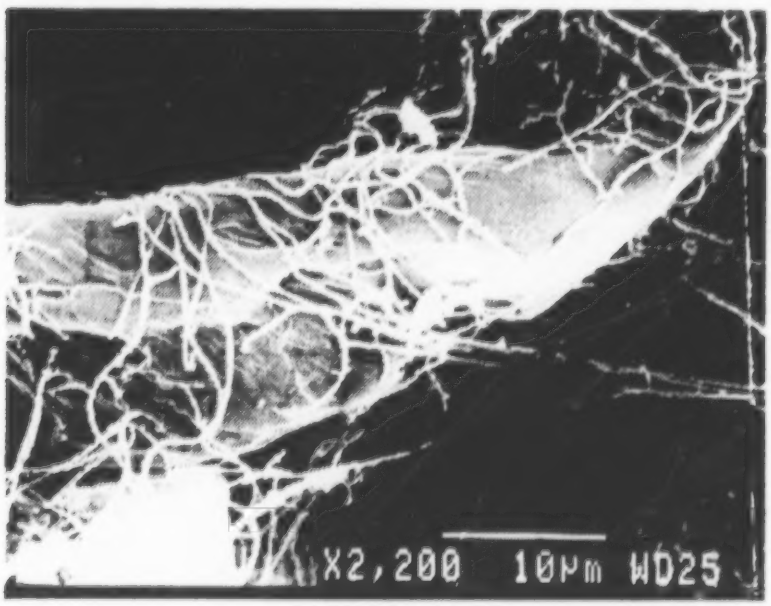

Figure 1. The filamentous bacterium, *T. curvata*, grows on the surfaces of plant fibers, digests the cellulose, and uses the sugars as a carbon and energy source.

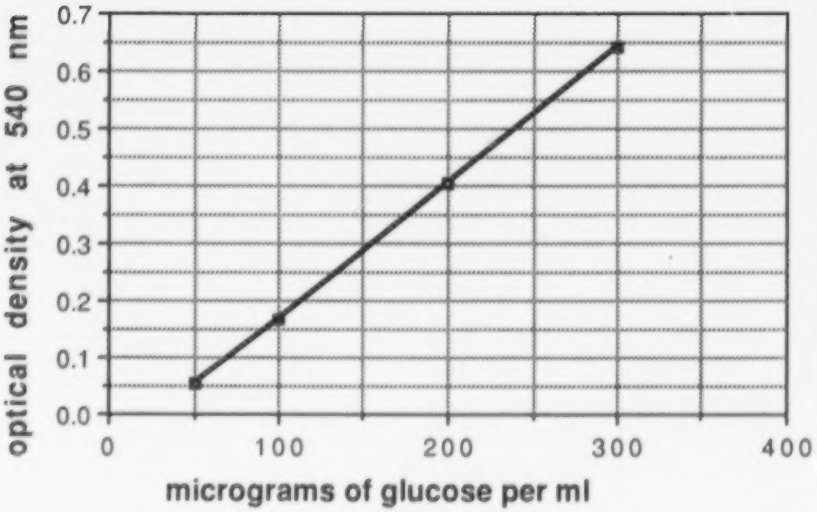

Figure 2. Typical glucose standard curve with the RSR.

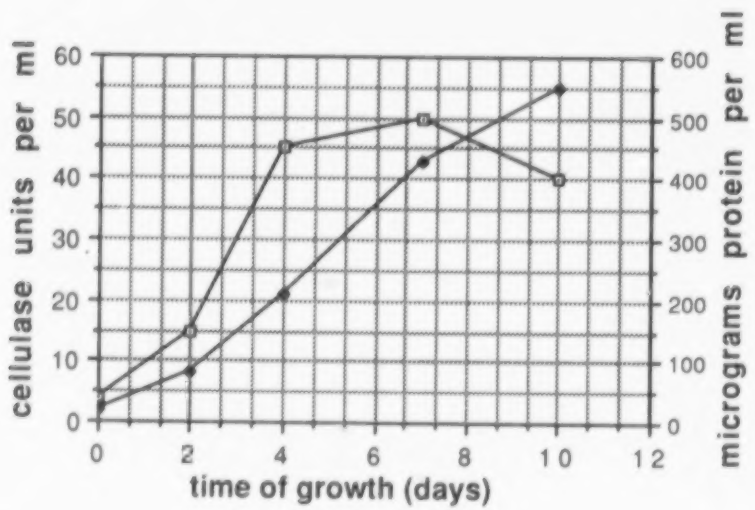

Figure 3. Typical results of cellulase (□) and protein (◆) from *T. curvata* culture.

SELECTION OF *ASPERGILLUS NIGER* MUTANTS WITH INCREASED AMOUNTS OF GLUCOSE OXIDASE

KEY WORDS: Glucose oxidase, *Aspergilluus*, Gluconate, Mutagenesis, Spores.

BACKGROUND:

The fungus *Aspergillus niger*, also known as common black bread mold, is used to manufacture commercially important products. One of these products is the organic acid gluconate, which is formed by the oxidation of glucose, a simple sugar. The oxidation reaction that produces gluconate is carried out by an enzyme in the fungus called glucose oxidase. This enzyme is sold commercially for clinical determination of glucose levels in biological fluids, desugaring eggs prior to dehydration, and removing oxygen from fruit juices packaged in bottles and boxes.

Using current fermentation technology, it is not possible to make glucose oxidase without also producing gluconate. The amount of gluconate produced presents a problem since industry cannot make enough glucose oxidase to meet the market demand, but currently produces more gluconate than it can sell. Isolation of a new strain of *A. niger* that produces more glucose oxidase would increase the availability of this useful enzyme, produce greater profits for the manufacturer, and not require any increase in production costs.

The traditional method of producing microbial strains with desirable traits for biotechnology is **mutagenesis** followed by **selection**. To start this process, a strain of the organism that already has some of the desired characteristics is used. This strain is subjected to either chemical or physical agents which cause changes in the genetic material of the organism. These agents are called **mutagens**, meaning that they produce changes called **mutations**. Some mutations have no effect and are not detected. Some mutations cause a change so severe that the changed organism dies; these are called lethal mutations. Other mutations can produce changes that are desirable, such as increased production of the glucose oxidase enzyme. Producing mutants (mutagenesis) is relatively easy; the difficult part of the process is finding desirable mutants through selection. It is normally necessary to subject millions or billions of organisms to mutagenesis to find one that has the desired characteristics. Finding that one organism is often like looking for a needle in a haystack.

My colleagues and I have published a method for mutagenesis of *A. niger* and selection of a class of mutants with increased amounts of glucose oxidase. The mutagen was nitrous acid, a chemical that modifies and changes DNA. We reasoned that fungi that produce more glucose oxidase would make the growth medium acidic faster than the starting organism or other types of mutants. After mutation, we grew the mutated organism on a solid medium containing glucose and methyl red. This latter compound is yellow in neutral solutions, but turns red as the solution becomes more acidic. After 5 days of growth, occasional colonies of an organism were observed surrounded by a red zone. These organisms were purified and a high proportion of them were found to contain higher levels of glucose oxidase than the starting strain.

STATEMENT OF PROBLEM:

To produce and select mutants of *Aspergillus niger* with increased ability to ferment glucose to organic acids.

METHODS AND MATERIALS:

Obtain a strain of *A. niger* from a Culture Collection or from a local university or college. Alternately, you can write to the author for a culture and a copy of the appropriate publication describing the original work and containing the recipes for all the necessary growth

media and solutions. Sterile technique must be employed in order to grow and harvest *A. niger* spores. If an agar plate appears to have more than one type of organism growing on it, do not open it. **Do not take chances with foreign microbes**. *A. niger* grows best in an incubator at 25°-30°C in the dark. If you do not have an incubator, plates of *A. niger* can be grown at room temperature in a drawer or wrapped in aluminum foil. When grown on an agar plate, the *A. niger* will slowly fill the plate and then begin to form black spores as the sugars are consumed. After 10-14 days at 25°C, the plate will be covered with black spores. Harvest the spores by washing the surface of the plate with a small volume (about 10 mL) of sterile water containing a small amount (0.025%, about 1 drop per 100 mL) of Triton X-100, a common laboratory detergent. If a spectrophotometer is available, the concentration of spores can be determined, an absorbance at 650 nm of 0.15 indicates a solution of 7.5×10^5 spores per mL. We used 2 mL of a spore solution at this concentration, but if it is not desired to determine the frequency of mutagenesis, quantitation is not necessary to continue the experiment.

To revive the spores, incubate them in the washing solution in a sterile centrifuge tube at room temperature for 3 hrs. Pellet the spores by spinning in a table-top centrifuge for 5-10 mins. Pour off the supernatant fraction and resuspend the spores in 1.8 mL of 0.1 M sodium acetate buffer (pH 4.4). Wearing plastic gloves, add 0.2 mL of sodium nitrite solution (5 mg per mL). This addition generates the mutagenic agent, nitrous acid, in the sodium acetate buffer. **Do not allow nitrous acid to come into contact with your skin. Throw gloves into trash and do not reuse.** Incubate the spores at room temperature with occasional shaking for 2 hours. Pellet the spores by centrifuging again and, wearing plastic gloves, carefully pour the supernatant fraction into a sink. Rinse the sink with water. Resuspend the spores in 50 mM sodium phosphate buffer (pH 7.0), harvest the spores by centrifugation, and repeat the neutral wash once more. The spores are finally resuspended in 2 mL of carbohydrate-mineral salts medium, and 50μl (0.05 mL) amounts are added to the surface of solid carbohydrate-mineral salts medium containing 0.001% methyl red (Markwell *et* al, 1989). The spores are spread across the surface of the medium with a bent, sterile glass rod. Incubate the plates at 25°C for 5 days. Mutant colonies able to rapidly ferment the glucose and produce more acid than the original strain will be surrounded by a pink or red zone. If the initial spore concentration was measured with a spectrophotometer, the number of rapidly fermenting mutants can be compared to the number of spores applied to the plates.

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ALTERING GENETIC CHARACTERISTICS

TRANSFORMATION OF CYANOBACTERIA USING A PLASMID SHUTTLE VECTOR

KEY WORDS: Cyanobacteria, Plasmid, Transformation.

BACKGROUND:

Cyanobacteria are a diverse group of photoautotrophs which evolve oxygen during photosynthesis in a manner similar to the chloroplasts of higher plants. The biology of cyanobacteria has been comprehensively reviewed in a number of current sources (Colowick and Kaplan, 1988, and Carr and Whitton, 1982). Because they are prokaryotes and unicellular, they are easy to maintain in the laboratory and have a rapid generation time. Thus, cyanobacteria have been used as simple model systems to provide spectacular advances in our understanding of numerous mechanisms which also apply to higher plants including: 1) the structure and/or functioning of photosynthesis components; 2) the biochemistry and regulation of atmospheric nitrogen fixation; 3) complementary chromatic adaptation, the process whereby certain cyanobacteria adjust photosynthetic pigment synthesis to changes in light quality and intensity; and, 4) the metabolism and infection of cyanobacteria by temperate and virulent cyanophages.

Most recently, specific members of two genera of cyanobacteria (*Synechococcus* and *Synechocystis*) which are naturally transformable have been employed to provide a valuable molecular approach to examination of these mechanisms. In the process of transformation, the genetic information of an organism may be manipulated by the introduction of new or altered DNA nucleotide sequences into a viable cell. The expression of foreign genes in cyanobacteria may also have important applications such as recent research involving the expression of mosquitocidal toxins in cyanobacteria which may be used to control disease-carrying mosquitoes by killing larvae which feed on genetically altered cyanobacteria (Helvering, 1989 and Angsuthanasombat and Panyim, 1989). It is difficult to overestimate the potential of transformation which is providing an experimental tool that is expanding interest in the use of these organisms in biological studies and biotechnology.

Transformation of cyanobacteria is currently being performed in two ways. Chromosomal transformation depends upon the recombination of internalized donor DNA (linear or nonreplicating plasmid) with homologous chromosomal DNA in the recipient cell. Plasmid transformation involves the introduction of a shuttle plasmid vector, which carries both cyanobacterial and *E. coli* origins of replication enabling maintenance of the plasmid in either species. In both types of transformation, the donor DNA contains a drug resistance gene which provides a powerful means of selection of recombinant cells.

The use of plasmid shuttle vectors for transformation provides several advantages. One advantage is that DNA which has been introduced into a cell may be easily reisolated and mapped or sequenced to permit the correlation of an altered phenotyepe with a specific mutagenized DNA sequence. In addition, the plasmid vectors are capable of being maintained in *E. coli* which has a shorter generation time (approximately twenty min) and for which relatively simple protocols for high yields of plasmid DNA have been developed. In addition, some of the shuttle vectors (such as pTNTV and pPGV5) incorporate special features such as strong promoters for enhanced expression of cloned genes and/or a temperature-sensitive repressor which permits temperature-dependent expression of mutant genes which might be detrimental to the cell if continuously expressed (Nahreini, Brad, and Vann, 1988).

STATEMENT OF PROBLEM:

In this experiment we will perform a cyanobacterial transformation using a shuttle vector. Recombinants will be selected based on their plasmid encoded resistance to an antibiotic. The number of cells exposed to the plasmid DNA will be determined and the

number of recombinants obtained will be counted to determine the efficiency of transformation.

This experiment will familiarize students with the basic methodology of cyanobacterial transformation. Further transformations may be performed using the plasmid vectors to introduce a foreign or altered DNA sequence.

METHODS AND MATERIALS:

A. Equipment/supplies Required:

autoclave, or means of sterilization of media
low speed centrifuge and sterile centrifuge tubes to pellet cells
fluorescent light strip
lighted incubator at 34°C (beneficial but not required)
shaker to grow cells (beneficial but not required)
sterile flasks with plugs, petri dishes, pipets
good quality water (deionized and may need to be put over charcoal to remove organics)
hemocytometer to count cells (beneficial but not required)
BG-11 liquid media and plates; top agar (beneficial but not required)

B. Plasmids and Cyanobacterium:

This experiment deals with the transformation of cyanobacterium *Synechococcus* PPC 7942 (also called *Anacystis nidulans* R2) which may be obtained from: 1)Pasteur Culture Collection, 75724 Paris Cedes 15, France, or 2) Culture Collection of Algae at the University of Texas, Austin, TX 78712. Appropriate plasmids to transform into this species include: pUC 104 or pUC 105 (6): pSG111 (7); pECAN 1 (8); pPLANB 1 and pPLANB 2 (8,9); pTNTV and pPGV5 (5). These plasmids may be isolated from bacteria using standard techniques or the DNA may be isolated from cyanobacteria (Colwick and Kaplan, 1988).

Contact local Universities for advice, plasmids, and for strains. Once local sources are exausted, the following individuals employed in biology/botany departments in universities have expertise in cyanobacterial transformations and might be contacted for cyanobacterial plasmids and strains or for advice: C. Vann, Ball State University, Muncie IN; S. Barnum, Miami University, Oxford, OH; H. Pakrasi, Washington University, St. Louis, MO.; G. Bullerjahn, Bowling Green State University, Bowling Green, OH; S. Golden, Texas A&M University, College Station, TX; L. Sherman, Purdue University, W. Laffayette, IN.

C. Protocol:

Prepare in advance: Sterile BG-11 media - 50 ml in flasks; BG-11 agar plates; growing culture of cyanobacteria.

Day 1: Inoculate a 50 ml culture of BG-11 with approximately 2-3 ml of cyanobacteria from a stock culture.

Day 3: Harvest the cells by 5 min centrifugation. Resuspend the cells in BG-11 and centrifuge, repeating 2-3 times to wash the cells. Resuspend the final pellet in approximately 5 ml of BG-11. Four hundred μl of the resuspended cells should be placed in a tube and approximately 0.2 - 1.0 μg of plasmid DNA added. A negative control should be included which contains no DNA. The cyanobacteria should be incubated with the DNA several hours to overnight (it may aid efficiency of transformation to cover the tubes so no light is available and to gently shake the tubes).

Day 4: Serially dilute both the control and transformed cells in BG-11 covering a range of 1X to 1/250X. The control should also be diluted to 10^{-5} and 10^{-6}. Plate 100 μl of each dilution on separate plates, either spreading the cells with a sterile spreader or by adding the cells to 4 ml of melted cooled BG-11 top agar. All plates are placed upside down about 1 foot below fluorescent lights.

Day 5: After 6-24 hours, which allows time for resistance to be expressed, antibiotic is added under all of the plates by lifting the media with a sterile pipet tip except for the 10^{-5} and 10^{-6} dilutions of the control, which are used to estimate the initial concentration of cells. A 100X antibiotic stock solution is added under the agar or in a top agar to give the final desired concentration (usually ampicillin to a final concentration of 0.5 μg/ml). The plates are placed back under the lights at either room temperature or at 34°C, colonies are counted after 7-14 days, and the percentage of transformed colonies is determined.

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CLONING AND MEASURING THE ACTIVITY OF BACTERIAL PROMOTERS

KEY WORDS: Promoters, Chlorampenicol Resistance, Chloramphenicol - Acetyltransferase, Vector, Recombinant DNA Cloning.

BACKGROUND:

The kinds of proteins needed by a cell vary throughout its life. It uses some proteins continuously, others sporadically; it requires a few in large amounts, others in much smaller quantities. Clearly a cell must have some means of regulating both the timing and quantity of protein synthesis. Probably the most important point of regulation is at the earliest stage of the flow of genetic information. The complex enzyme, RNA polymerase, copies the DNA code into RNA. Before it starts the copying process (transcription), it must first bind to the DNA at a region in front of a gene. This region is called the gene's promoter. If RNA polymerase binds readily to a promoter, the gene is transcribed frequently and the protein it encodes tends to be present in large quantities in the cell. Such a promoter is called "strong." If RNA polymerase binds poorly to a promoter (a "weak" one), there is little transcription of the gene and little of the protein made. Differences in strength among promoters are due to differences in the DNA sequences that make up the promoters. The genetic machinery of the cell--including RNA polymerase--varies subtly from species to species. Because of this difference, a strong promoter from one species may be weak or even inoperative when placed in a distantly related species.

Promoter strength and species variation are important considerations in biotechnology. Often it is desirable to engineer a cell to make large quantities of some useful protein. When a foreign gene encoding the protein is introduced into the cell, the gene's own promoter may not function well and the protein may be inadequately produced. It may be necessary to alter the base sequence of the promoter or to place an entirely new promoter in front of the gene.

Promoters can be isolated and studied through the use of promoter-probe cloning vectors. Such a vector contains a gene with no promoter. The gene encodes a protein that can be easily assayed. A cell containing the vector alone would make very little of the protein. However, if we insert a fragment of foreign DNA into the plasmid immediately in front of the gene, any promoter present on the insert would turn the gene on. The strength of the promoter could be measured by the amount of the assay protein produced. In this project, promoters from the bacterium *Bacillus subtilis* are cloned into *B. subtilis* or the distantly-related *Escherichia coli*. The promoters turn on a chloramphenicol-acetyltransferase (CAT) gene, rendering the host cell resistant to the antibiotic chloramphenicol. The strength of the promoter is assayed by the level of antibiotic resistance. Individual promoters can be tested in both *E. coli* and *B. subtilis* in the same cloning vector.

STATEMENT OF PROBLEM:

To clone *Bacillus subtilis* promoters; to assay for their level of activity in *Escherichia coli* and *Bacillus subtilis*; to compare activity of individual promoters in these two organisms.

METHODS AND MATERIALS:

The project is accomplished through the following steps. (All bacterial cell lines and vectors may be obtained at no cost from the author.)

1. Isolation of *B. subtilis* chromosomal DNA--This may be done by the method of Marmur (1961). If necessary, I will supply a detailed protocol upon written request. Most DNA isolation procedures require the use of organic solvents. Gloves, goggles, and lab coats should be worn. A shaking incubator and bench top centrifuge are required.

2. Isolation of pCPP-3 and pCPP-4 (Band et al., 1983) plasmid DNA from *E. coli*--The small scale, rapid methods presented in Maniatis (1982) are adequate. A microcentrifuge is required. These plasmids can replicate in either *E. coli* or *B. subtilis*. They render either organism resistant to the antibiotic neomycin. Each plasmid also carries a promoter-less chloramphenicol resistance (CAT) gene with a unique restriction enzyme cleavage site just upstream from the gene. Promoters are inserted into this site to turn on the CAT gene.

3. Digestion of chromosomal DNA and plasmid with appropriate restriction enzyme (*Bam* HI or compatible enzymes for pCPP-3, *Eco* RI for pCPP-4) and ligation with T4 DNA-ligase. Buffers and reaction protocols are supplied by the manufacturers (Bethesda Research Labs, New England Biolabs, International Biotechnologies, etc.).

4. Transformation of host with ligation mix--For *E. coli*, transformation-competent cells are best prepared by the $CaCl_2$ method in Maniatis (1982). For *B. subtilis*, the competent cell preparation procedure of Hardy (1985) is far easier than his protoplasting technique; the former technique will work if a restriction-minus host is used. Transformants of either species are selected on 10 μg/ml chloramphenicol (Sigma Chemical Company). Incubators and water baths are required.

5. Assaying for chloramphenicol resistance--A transformant can be tested for level of resistance, which will range from 10-2000μg/ml in *E. coli* and somewhat less in *B. subtilis* (Band et al., 1983). No special equipment is needed. Spectrophotometric assays are more precise, but more difficult and require a good spectrophotometer.

6. A plasmid containing a cloned promoter can be isolated from one species and tested in the other by the methods above. These vectors work in either species. Other possible directions: If funding and a cooperating lab are available, a student could study the nucleotide sequences of promoters to determine what makes a promoter strong for either of the species; if the colorometric CAT-assay method is developed, a student could study the timing of CAT synthesis for given promoters, i.e., the relative strength of promoters at various points in the culture's growth curve.

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PRODUCING GENETICALLY ENGINEERED BACTERIA VIA TRANSFORMATION WITH RECOMBINANT DNA PLASMIDS

KEY WORDS: Transformation, Plasmid, DNA, Recombinant DNA, Genetic Engineering.

BACKGROUND:

The introduction (transformation) of eukaryotic DNA into bacteria via recombinant plasmids (independent, circular pieces of DNA found naturally in many bacteria) has been one of the major developments in molecular biology. With the ability to produce unlimited quantities of the eukaryotic DNA sequences introduced into the bacteria (gene cloning), scientists have been able to understand the functioning and structure of eukaryotic genes at the molecular level including their specific base sequence. It has also facilitated the development of genetic engineering with all of its potential for creating useful organisms and correcting genetic defects.

In principle, making recombinant plasmids is easy (Watson *et al*, 1983, and Curtis and Barnes, 1985). The plasmid DNA is usually cut in one spot with a restriction enzyme (proteins that recognize specific sequences within DNA and cause breaks at these sites) and joined (ligated) to eukaryotic DNA cut with the same restriction enzyme. A larger, recombinant plasmid with a piece of eukaryotic DNA inserted at the restriction target site is the desired result (see fig. 1). The recombinant plasmids are then introduced back into a bacterial host via transformation. The introduced DNA may be random restriction fragments from the organism of interest, or more specific genes isolated by various means.

In practice, making recombinant plasmids is more complex (Maniatis *et al*, 1982, and Watson *et al*, 1983). The major difficulty is to distinguish between bacteria with plasmids that contain sequences of eukaryotic DNA and those with plasmids that have simply recircularized without the insertion of any DNA (see fig. 1). However, a number of plasmids, such as pUC18, have been developed to make it relatively easy to select recombinants genetically. Most of these plasmids rely on what is called insertional inactivation. These plasmids contain an easily identifiable functional gene, such as the *lacZ* gene. The plasmid is cut in the middle of this gene to insert the eukaryotic DNA. Thus, recombinants will lack the functional gene, since a DNA fragment with a completely different function has been inserted, while recircularized plasmids will still have activity (see fig. 1). For example, when pUC18 plasmids with a functional *lacZ* gene are transformed into a *lacZ* - bacterium, the bacteria will grow into a blue colored colony on media containing X-gal, while the colonies containing recombinant plasmids are white. Alternatively, the colonies will be brick red on MacConkey's agar and those without a functional *lacZ* gene will be clear. The plasmids used for this type of experiment usually also contain an antibiotic resistance gene. This gene acts as a convenient way to select transformed cells, since only bacteria transformed with the plasmid can grow in antibiotic containing media.

STATEMENT OF PROBLEM:

The objective of this project is to develop novel plasmids each containing a fragment of eukaryotic DNA (yeast, plant, etc.) and then introduce these recombinant plasmids via transformation into *E. coli*. The bacteria which obtain a plasmid will have new genetic properties. Thus, a unique, "genetically engineered" organism will have been developed! These *E. coli* will each contain fragments of DNA from the eukaryotic donor which could potentially be studied in further research.

METHODS AND MATERIALS:

The procedure for this experiment needs to be carried out over several days. It will involve two main steps: creating recombinant plasmids, and transforming appropriate bacteria with the plasmid DNA. A high speed centrifuge or microcentrifuge, a refrigerator/freezer, an adjustable micropipet or assorted glass micropipets, a balance and pH meter for making solutions and an autoclave are required equipment. An incubator with a shaker would be helpful. This equipment is all standard at any college biology department or analytical/testing lab. Students in the Cleveland are welcome to use my lab at Baldwin-Wallace.

Step 1: You will use a restriction enzyme to cut plasmid and eukaryotic DNA. At Baldwin-Wallace, we use pUC18 plasmid DNA and the enzyme Pst I purchased from Sigma or U.S. Biochem. We generally use eukaryotic DNA that we have isolated ourselves; however, commercially purified DNA is available. After the plasmid and eukaryotic DNA has been cut, it is precipitated with ethanol and ammonium acetate to separate the DNA from the restriction enzyme. The DNA is then joined together with T-4 ligase. When doing a ligation reaction, it is important to have the right proportion of plasmid DNA to eukaryotic DNA. What counts is the ratio of free ends per unit weight (μg) of DNA. We use 7 μg of plant DNA for each μg of pUC18. Most other eukaryotes will be in this range, but you may want to try several different ratios.

Step 2: To complete the transformation step, you will need to obtain or produce competent *E. coli* DH5-alpha cells (this is a K-12 strain of *E. coli* which can accept the pUC18 plasmid). Frozen competent cells can be purchased from BRL. We have also followed Dagert and Ehrlich's competency treatment (Dagert and Ehrlich, 1979) with success. It is helpful to do both positive and negative transformation controls. After being transformed the DH5-alpha cells need to be aseptically spread on selection plates (Maniatis *et al*, 1982) and incubated at 37°C for 24 hours to obtain results. You should be able to interpret the results of the control plates, calculate the number of transformants per μg of DNA for all plates, and the percentage of white (recombinant) colonies.

General protocols for Steps 1 and 2 can be found in the fourth reference listed below, as well as formula for media and solutions. I will also be happy to send you the specific protocol we follow, and answer any questions.

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Figure 1: Use of insertional inactivation of the lacZ gene to screen for transformed *E. coli* with a recombinant plasmid.

TRANSFORMATION OF *SACCHAROMYCES CEREVISIAE*

KEY WORDS: Transformation, Yeast, Plasmid, Genetic engineerinng Antibiotic Resistance.

BACKGROUND:

DNA was shown to be the genetic material of prokaryotic cells by Avery, McCloud and McCarty's (Hartl *et al*, 1988, and Lewin, 1990) transformation experiments in 1944. They demonstrated that one strain of Pneumoccocus could acquire an inheritable characteristic from the DNA of another strain. It was not until 1978 that Hinnen, Hicks and Fink (Bolstein and Davis, 1982) successfully performed similar transformation experiments with a eukaryotic organism, the yeast *Saccharomyces cerevisiae*, thus demonstrating that DNA is the genetic material of eukaryotic organisms as well.

The ability of organisms to acquire inheritable characteristics by transformation makes it possible to change the phenotype of an organism in a specific way by transforming it with an appropriate DNA molecule. This is the basis of the idea that genetic defects in humans could be cured by transforming cells with a functional copy of a defective gene. In the experiment described below, a yeast strain which cannot grow without uracil because of a mutation in a gene coding for an enzyme involved in uracil biosynthesis will be transformed with a plasmid carrying a functional copy of the gene. The yeast which are successfully transformed will have acquired the ability to grow in the absence of uracil.

STATEMENT OF PROBLEM:

The purpose of this experiment is to transform the eukaryotic organism *Saccharomyces cerevisiae* and thus to confirm that DNA is the genetic material.

METHODS AND MATERIALS:

Any yeast strain carrying the non-revertable ura3-52 allele of the URA3 gene is suitable for this experiment. Yeast strains can be obtained from the Yeast Genetics Stock Culture Center, Donner Laboratory, University of California, Berkeley, CA 94720. Any plasmid carrying a wild type copy of the URA3 gene is suitable for use in this experiment. Restriction maps and information about three such plasmids--YEP24 (a high copy number plasmid), YRP17 (a low copy number plasmid) and YIP5 (a plasmid which integrates into chromosomes) are available in the free New England Biolabs Catalog from New England Biolabs, 32 Tozer Road, Beverly, MA 01915-5510. Due to the availability of these plasmids, they must be obtained from the lab that originally constructed the plasmids or other yeast researchers such as our lab. In addition, a microscope, a hemocytometer, sterile centrifuge tubes, petri dishes and equipment for plating micro-organisms are required for this experiment. Microliter amounts of reagents and DNA can be measured with inexpensive microcapillary pipets (For example Fisher #21-164-2A).

Protocol for LiOAc Transformation of Yeast (Campbell and Duffus):

1. Grow an overnight culture of the strain to be transformed in YEPD.
2. In the evening, count the cells and inoculate 200 ml YEPD (for 5-10 - transformations) to have a cell titer of $1x10^7$ cells/ml the next morning. In order to do this, you must know the generation time of the particular strain you are using which can easily be estimated by counting cells at various times during log phase growth.
3. Harvest the cells by centrifugation in a table top centrifuge (maximum speed for about 2 min.)
4. Resuspend the cells in 30 ml sterile water and repeat the centrifugation.
5. Resuspend the cells in 5 ml LiOAc buffer. Repeat the centrifugation.

6. Resuspend the cells in 5 ml (use 1 ml per transformation) LiOAc buffer/1M sorbitol (mix LiOAc buffer and 3 M sorbitol 2:1).
7. Place the tube in an incubator shaker for 1 hour at 30°C.
8. Transfer 1 ml each into sterile conical tubes and spin for 2 min. Remove the supernatant carefully with a pasteur pipet.
9. Mix 5-25 μl of the plasmid DNA (1-10 μg) and 25 μl sheared Salmon sperm DNA (1 mg/ml in TE) with 70 μl LiOAc buffer and 50-60 μl 3M sorbitol (depending on the volume of the DNA solution).
10. Resuspend the cells in this mixture.
11. Incubate for 30 min. at 30°C without shaking.
12. Vortex briefly, then add 1 ml LiOAc/PEG buffer and incubate for another 20 min at 30°C.
13. Incubate for 5 min at 42°C.
14. Plate 200μl per plate on minimal media without uracil. Plates with uracil and yeast cells to which no DNA has been added should also be plated as controls. Incubate at 30°C.
15. After 3-4 days, look for colonies growing up from the background of dead cells on the plates.

If more sophisticated equipment is available, it is possible to recover the plasmids from the yeast strains, compare the transformation of linear and circular DNA, and determine what sequences on the plasmids are necessary for transformation.

Recipes:

LiOAc Buffer

100 mM lithium acetate
10 mM Tris-HCL pH 7.5
1 mM EDTA

LiOAc/PEG Buffer

40% PEG 3500
100 mM lithium acetate
10 mM Tris-HCL pH 7.5
1 mM EDTA

Sheared Salmon Sperm DNA

1 mg/ml salmon sperm DNA in TE
Suspend the DNA in TE (let it sit overnight)
Then force solution through a series of needles from 18-25 gauge to shear DNA.

YEPD media

5 g Yeast Extract 500 ml=20-25 plates
10 g Peptone

Add 5.0 mls 100x URA stock solution (2 g uracil in 100 mls of water)
Add 494 mls water
Autoclave 25 min
After autoclaving, add 50 mls 50% glucose.

Yeast Minimal Media

Yeast Nitrogen Base w/o Amino Acids or Ammonium Sulfate (from Difco Labs, Detroit, MI)	0.85 g
Ammonium Sulfate	2.5 g
Agar	7 g

Autoclave then add the following filter sterilized solutions.
+50 mls of 50% glucose
+5 mls of 100x stock solutions of appropriate amino acids depending on the strain used
+5 mls of 100x URA stock solution for plates with uracil

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TRANSFORMATION OF THE BAKER'S YEAST SACCHAROMYCES CEREVISIAE WITH PLASMIDS CARRYING BACTERIAL NEOMYCIN RESISTANCE GENES

KEY WORDS: Transformation, Yeast, Plasmid, Vector, Neomycin.

BACKGROUND:

The baker's yeast *S. cerevisiae* was first used as a host organism in 1978 (Hinnen *et al.* 1978) and has since then attained much attention as a host for the production of pharmaceuticals and for studying fundamental questions in basic research (for a review see Kingsman *et al.* 1985). Whereas in the latter case laboratory strains carrying mutations that can be used for the selection of transformants by complementation with wild type genes (e.g. amino acid auxotrophies) are employed, in the fermentation industry "wild" strains are used that are mostly diploid or polyploid and do not possess auxotrophic deficiencies. Transformation of these strains requires a dominant selectable marker such as an antibiotic resistance. Resistance towards aminoglycoside antibiotics as neomycin and kanamycin (and G418 in eukaryotes such as yeast) is mediated by bacterial resistance genes. These have been isolated from different strains. The neomycin resistance genes most widely used in bacterial and eukaryotic transformation are derived from transposons Tn903 or Tn5. They code for aminoglycoside-3'-phosophotransferase type I and type II, respectively. Whereas in plant cells and mammalian cell lines the Tn5 gene is predominantly used for selection of transformants, in yeast the Tn903 gene seems to be more suitable for dominant transformation (Lang-Hinrichs *et al.* 1989).

STATEMENT OF PROBLEM:

To establish a vector and transformation system for laboratory and commercial yeasts based on bacterial neomycin resistance genes and to use this system to compare the effect of two different genes coding for the same enzyme activity on yeast transformation.

MATERIALS:

Plasmids for yeast transformation and yeast strains are available from the American Type Culture Collection, e.g. yeast strains SHY1, SHY2, SHY3 or SHY4 (ATCC 44769 - 44772) and plasmid pYE13G (ATCC 37276) containing the Tn903 neo^R gene on a yeast/*E. coli* shuttle vector. The Tn5 neo^R gene is available e.g. on plasmid pSV3-neo ATTC and has to be cloned onto a yeast/*E. coli* shuttle vector such as YEp13 (ATCC 37115). The antibiotic G418 is commercially available from GIBCO or Sigma (as "Geneticin").

Note: Gloves should be worn when using this antibiotic and care should be used when handling it.

EQUIPMENT:

The project requires the equipment of a common molecular biology/microbiology laboratory. Except for the radioactive *in vitro* assay which is not essential for the project, no other specialized equipment is needed.

CAUTION:

All experiments involving recombinant DNA and/or cells treated with recombinant DNA have to be carried out according to the recombinant DNA guidelines!

METHOD:

To examine transformation efficiency and resistance mediated by the bacterial genes in yeast cells, plasmids are used which consist of the respective bacterial gene and a common yeast vector (YEp13) containing a yeast replication origin and a yeast gene (a leucine synthesis gene which complements the yeast leu2 mutation) as selectable marker (plasmid pYE13G for the Tn903 gene). Construction of the plasmid consisting of YEp13 plus the Tn5 neo^R gene from plasmid pSV3-neo is done following the protocols for molecular cloning as detailed in Maniatis *et al.* (1982) or Berger and Kimmel (1987). Plasmid isolation (Birnboim and Doly 1979): Plasmid-carrying *E. coli* strains are grown in 10 ml LB medium (1% peptone, 0.5% yeast extract, 0.5% NaCl pH 7.2, ampicillin is added at 50 μg/ml) at 37°C overnight. Cells are pelleted by centrifugation and resuspended in 250 μl 10 mM Tris, 1 mM EDTA pH 8.0 (T.E. buffer). Cells are lysed by addition of 0.5 ml 0.2 N NaOH, 1% SDS and incubation at room temperature for 5 min. Chromosomal DNA is precipitated by addition of 2 ml 0.5 M NaCl, 1 M sodium acetate and incubation for 15 min at room temperature followed by centrifugation for 15 min at 12000 x g. Plasmid DNA is precipitated from the supernatant by adding 5 ml ethanol, followed by incubation at -20°C for 15 min and centrifugation as above. The DNA pellet is allowed to dry and is resuspended in 0.1 ml T.E. buffer.

These plasmids are transferred into yeast cells (e.g. SHY3) by lithium acetate transformation.

Yeast transformation (Ito *et al.* 1983): The yeast strain is grown in an overnight culture to stationary phase and 50 to 100 μl of this culture is inoculated into 100 ml YE (0.5% yeast extract, 2% glucose) medium and grown for 16 h at 26-30°C (the cells should by then have grown to a density of 5 x 10^7/ml). Cells are pelleted, washed twice with T.E. buffer, resuspended in 1.5 ml 0.1 M lithium acetate and incubated with shaking at 26°C for 1 h. 20 μl plasmid DNA and 50 μg denatured salmon sperm DNA (carrier DNA) is added to 0.3 ml cell suspension. After addition of 0.7 ml 50% polyethylenglycol 6000 and careful mixing, cells are incubated for 1 h at 26°C. 100-200 μl are then plated onto minimal medium (YNB: 0.67% DIFCO yeast nitrogen base, 2% glucose, 1.5-2% agar, amino acids are added at 40 μg/ml as required for the strain used). Transformants appear after 3-4 d at 26-30°C. To determine the resistance level conferred by the different genes, transformed colonies are then inoculated onto media (YE agar) containing the antibiotic G418 at different concentrations (between 200-500 μg/ml).

Use the yeast vectors described above to directly select for $G418^R$ transformants in both laboratory and industrial yeast strains. (e.g. commercially available baker's yeast, wine yeast or brewing yeast). Transformation is done as described above, except that a regeneration time of 4 h in YE medium is allowed before the cells are plated onto YE medium containing 200 - 300 μg/ml G418 (Sakai and Yamamoto 1986). An alternative method has been described by Webster and Dickson (1983).

Compare transformation frequencies and resistance levels obtained with the different plasmids.

Colony hybridization studies could also be done to demonstrate the existence of the DNA in the yeast

A further characterization of resistant transformants can be achieved by preparing cell-free extracts and determining the *in vitro* enzyme activity using a radioactive assay (Ozanne *et al.* 1969, Lang-Hinrichs *et al.* 1989).

To improve the expression of the bacterial genes in yeast, the insertion of a homologous promoter at the 5'end of the bacterial genes is possible (Lang-Hinrichs *et al.* 1989).

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These books and articles are available through university libraries. For reprints of specific articles or for correspondence, write to Dr. Christine Lang-Hinrichs.

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TRANSFER OF GENETIC INFORMATION BETWEEN BACTERIA

KEY WORDS: DNA, Conjugation, Plasmid, Antibiotic Resistance.

BACKGROUND:

Bacteria contain one haploid, circular chromosome often referred to as chromosomal DNA (deoxyribose nucleic acid). It is this piece of genetic material that dictates the biological activities and characteristics of the organism. In addition to the chromosomal DNA, some bacteria possess an extrachromosomal piece of DNA termed episomal or plasmid DNA. Often contained within these DNA plasmids are genes which impart antibiotic drug resistance. In other words, bacteria which contain a drug resistance plasmid are able to grow in the presence of the drug, and cannot be killed by the specific antibiotic. This is why, in some cases, certain human bacterial infections must be treated with multiple antibiotics, referred to as antibiotic therapy. The effectiveness of using various antibiotics in improving growth rate and feed efficiency of domesticated farm animals, perhaps due to the suppression of subclinical bacterial infections, has resulted in the routine incorporation of these drugs into the diets of many animals (Gast and Stephens, 1985).

Bacteria can exchange genetic information (DNA) by three mechanisms:

1. Transformation - in which foreign DNA from a lysed (broken open) bacterial cell is incorporated (taken up) by another bacterial cell.

2. Transduction - in which a bacteriophage (virus) acts as a vector (source) carrying the bacterial DNA between two cells.

3. Conjugation - (sometimes referred to as mating) in which bacterial DNA is transferred from one cell (donor) to another (recipient) by direct cellular contact.

It is believed that the selective adaptation pressure exerted by the use of antibiotics in animal feed and the ability of bacteria to transfer this genetic resistance information both within and between species could possibly account for the drug resistance of human disease-causing bacteria.

STATEMENT OF PURPOSE:

To demonstrate and verify the transfer of genetic material from one organism to another.

METHODS AND MATERIALS:

1. Broth culture of *Escherichia coli* K_{12} (donor), which demonstrates transferable resistance to tetracycline.

2. Broth culture of *Escherichia coli* which is unable to ferment lactose due to a mutation in the lac operon (recipient) which is sensitive to tetracyline. **NOTE**: Use care when handling. **DO NOT MOUTH PIPET.**

3. Brilliant Green Agar (Difco, Detroit, MI) with 25μg/ml tetracyline (Sigma Chemical, St. Louis, MO) added after sterilization.

4. Antibiotic medium #3 broth (Difco).

5. Bacteriological loop.

6. Pipets (1ml).

7. Petri dishes (100 mm x 15 mm).

From each bacterial culture tube, transfer 0.1 ml of doner and 0.2 ml of recipient to a tube of antibiotic medium #3 broth and incubate at 37°C for 18-24 hrs. This is the conjugation tube.

Post incubation; from each tube, donor, recipient and the conjugation tube; streak for isolation onto Brilliant Green agar containing tetracycline and Brilliant Green agar without tetracycline for a total of 6 plates.

Incubate plates at 37°C for 18-24 hrs.

Post incubation; observe the colony types on each of the agar media and record results.

EXPECTED RESULTS:

E. coli ferments lactose and will produce yellow colonies on Brilliant Green agar. Since *E. coli* is resistant to tetracycline, it should grow and produce yellow colonies on both agar media with and without tetracycline.

The recipient can not ferment lactose and will produce red-green colonies on Brilliant Green agar. Since the donor without the DNA from *E. coli* (negative control) is sensitive to tetracycline, it should only grow on agar medium without the antibiotic.

Streak plates from the conjugation tube should contain yellow colonies (donor) and red-green colonies (recipient containing drug resistance from donor) on agar containing tetracycline.

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APPLICATIONS

OF

DNA ANALYSIS

PLASMID DNA ISOLATION AND CHARACTERIZATION

KEY WORDS: Plasmid, DNA, Electrophoresis.

BACKGROUND:

Plasmids are circular, extrachromosomal DNA elements found primarily in bacteria. They range in size from a few thousand to several hundred thousand nucleic acid base pairs. This still represents only a fraction of the DNA in the bacterial chromosome, which contains millions of base pairs of DNA. Plasmids are often characterized according to whether or not they can be spread from bacterium to bacterium by the genetic exchange mechanism of conjugation. Those that can be transferred are called conjugative plasmids, while those that cannot be exchanged are nonconjugative.

Plasmid DNAs often contain genes that confer specific phenotypes to the organisms harboring them. Among the characteristics encoded for by plasmid genes are: antibiotic resistance, antimicrobial toxins, pathogenic determinants, and cellular metabolic activities. The three bacterial species used in this exercise contain plasmids that confer resistance to the antibiotic ampicillin (*Escherichia coli*), are involved in the anabolic process of cellulose production (*Acetobacter xylinum*), and allow the catabolic breakdown of organic chemicals (*Pseudomonas putida*).

Genetic engineering technology has made use of plasmid DNAs in the development of cloning vectors. These are DNA molecules that can be recombined with DNA from other sources and reintroduced into a host cell where they are replicated. Because many plasmids used in cloning can replicate many times before cell division (high copy number plasmids), they allow for both the purification and amplification of any DNA sequence they contain. Plasmid cloning vectors have been used to isolate a variety of genes ranging from those involved in plant photosynthetic activity to human insulin production. The *E. coli* plasmid used in this exercise, pBR322, is a widely used cloning vector.

STATEMENT OF PROBLEM:

To compare the plasmid DNA content of three different bacterial species and to examine the relationship between plasmid content and cellular phenotype.

METHODS AND MATERIALS:

Strains and growth conditions

Escherichia coli HB101 containing pBR322 (L broth plus 25 μg/ml ampicillin at 37°C)

Acetobacter xylinum ATCC#23769 (H&S broth at 30°C)

Pseudomonas putida ATCC#33015 (Nutrient broth at 30°C)

Solutions

L broth: 10 g Bacto-tryptone, 5 g yeast extract, 10 g NaCl per liter

H&S broth: 20 g glucose, 5 g yeast extract, 5 g peptone, 3.3g Na_2HPO_4:$7H_2O$, 1.15g citric acid per liter

Nutrient broth: 3 g Bacto-beef extract, 5 g bacto-peptone per liter

Ampicillin Stock solution: 25 mg ampicillin/ml sterile distilled water

Lysing solution: 0.1 M Tris pH 12.5 (adjusted with 0.5 M NaOH), 3% SDS

Phenol:chloroform: mix equal volumes of phenol (ultrapure or redistilled) with chloroform and equilibrate with 0.2 volumes of 2 M Tris-HCl, pH 7.0

Dye solution: 0.1% SDS, 0.05% bromophenol blue, 30% glycerol, in water

Electrophoresis buffer: 0.089 M Tris, 0.089 Boric Acid, 0.002 M EDTA

Agarose gel: 0.7 g agarose in 100 ml of electrophoresis buffer

Ethidium bromide: 4 μg/ml in water

Methylene blue: 0.25 mg/ml in water

Plasmid Isolation Procedure

1. Grow an overnight culture of bacteria in 1 ml of appropriate broth. Pipet 0.5 ml into a 1.5 ml microfuge tube and collect cells by spinning in a microcentrifuge for 2 min.

2. Add 0.1 ml of lysing solution and vortex to resuspend cells. Place cells in a 65°C water bath for 30 min.

3. Add 0.1 ml of phenol:chloroform solution, vortex (this shears the chromosomal DNA), and spin for 2 min in the microfuge.

4. Allow the tubes to stand for 5 minutes. During this time, the denatured proteins may collect on the side of the tube. Take 25 μl of supernatant and transfer it to a new tube. Add 2.5 μl of dye solution.

Agarose Gel Electrophoresis

1. Load 20 μl of samples into the wells of a 0.7% agarose gel.

2. Electrophorese at 100 V for 1-2 hrs (depending on the size of gel), or until the bromophenol blue dye has migrated to within an inch of the end of the gel.

3. Stain gel with either ethidium bromide (stain 5 min, destain in water 5 min) or methylene blue (stain 15 min, destain in water) to visualize the DNA bands.

Note: The bacteria used in this exercise are nonpathogenic, but should be treated as such and handled using sterile technique. The phenol:chloroform solution is caustic (will cause skin burns) and should be handled with care. Ethidium bromide is a powerful mutagen and when handling you should always wear gloves. Gels stained with ethidium bromide must be viewed while illuminated with an ultraviolet light source. UV absorbing glasses or other forms of eye protection must be used when viewing gels. Special equipment such as a microcentrifuge, micropipets, electrophoresis units are necessary to do this exercise.

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ASSOCIATION OF BACTERIOCIN ACTIVITY WITH PLASMIDS IN *PEDIOCOCCUS*.

KEY WORDS: Bacteriocin, *Pediococcus* plasmid, Microbial inhibition.

BACKGROUND:

The pediococci are non-pathogenic lactic acid bacteria that have numerous uses in the food industry. Their primary commercial use is as starter cultures in the manufacture of fermented sausages such as pepperoni. Pediococci are also natural participants in vegetable fermentations, where their metabolism contributes to flavor, texture and color development as well as inhibiting growth of spoilage and pathogenic microorganisms.

Bacteriocins are small, molecular weight proteins secreted by bacteria that specifically inhibit or kill other bacteria. The genes for these antibiotic-like proteins are usually located on small extrachromosomal elements of DNA, better known as plasmids. Although bacteriocins generally inhibit bacteria very similar to those that produce them, some lactic acid bacteria have bacteriocins shown effective against gram-positive pathogens such as *Clostridium botulinum*, *Staphylococcus aureus* and *Listeria monocytogenes*. In fact, nisin, a bacteriocin produced by the cheese-making organism, *Lactococcus lactis*, has proven so effective against clostridia (while safe for humans) that it has been approved by the FDA for use in cheese spreads as a preservative agent.

This project is based on the work that first documented a bacteriocin from lactic acid bacteria (*Pediococcus*) that was effective against *L. monocytogenes* (Hoover *et al.*, 1988). In recent years, *L. monocytogenes* has become a major concern to the food industry and consumers. *L. monocytogenes* has been found to naturally occur in raw meat, seafood and dairy products. In susceptible people, listeriosis can be fatal.

STATEMENT OF PROBLEM:

To examine the bacteriocin-activity of pediococci against a variety of other microorganisms and to confirm that this inhibition is associated with a small molecular weight plasmid.

METHODS AND MATERIALS:

Cultures: *Pediococcus acidilactici* PO2 - A bacteriocin producer
Pediococcus acidilactici PO2K5
Leuconostoc mesenteroides 8293 - A strain which is sensitive to bacteriocin

Medium: APT broth (Difco)
APT agar (Difco)
Bacto-agar (Difco)

Screening for bacteriocin (Bac) production:

The method of Kekessy and Piguet (1970) was used. One loopful of a 16 h APT broth Bac-producing culture incubated at 32°C was spot-inoculated onto the surface of an APT agar plate and incubated at 32°C for 16 to 18 h. The agar was detached from the edges of the petri dish with a sterile spatula. The covered plate was then inverted and tapped sharply on a hard surface so that the agar fell into the lid. Those cultures tested for sensitivity to Bac were grown in APT broth at 32°C for 18 h (another general growth broth can be substituted for APT). A suspension of cells (0.5 ml) was added to a tube containing 7 ml of 45°C-tempered APT broth containing 0.7% agar, mixed, and poured over the surface of the inverted agar. Plates were incubated at 32°C for 16 to 20 h and examined for zones of inhibition.

PLASMID ANALYSIS:

Materials required:

Microcentrifuge and vials
Micropipetor and tips
37°C bath
UV light cabinet
Polaroid camera
red filter and film
gel apparatus and power unit

Chemicals:

sucrose, EDTA, Tris buffer, sodium dodecyl sulfate, sodium hydroxide, sodium chloride, chloroform, isoamyl alcohol, isopropanol, acetic acid, agarose, ethidium bromide, lysozyme

The technique used to obtain plasmid DNA for examination by agarose gel electrophoresis is based on the Anderson and McKay procedure (1983). The culture volume recommended for this protocol is 1.5 ml or 600 ml if a large scale preparation is done. The volumes for large scale preparations are in parentheses.

1. Resuspend the pelleted cells in 379 μl (30 ml) of 6.7% sucrose in 50 mM Tris-1 mM EDTA, pH 8.0.

2. Warm to 37°C.

3. Add 96.5 μl (7.5 ml) of lysozyme (10 mg/ml in 25 mM Tris, pH 8.0).

4. Incubate for 5 min at 37°C.

5. Add 48.2 μl (3.75 ml) of 0.25 M EDTA-50 mM Tris, pH 8.0.

6. Add 27.6 μl (2.25 ml) of 20% sodium dodecyl sulfate (wt/vol) in 50 mM Tris-EDTA, pH 8.0) and mix immediately.

7. Incubate for 5 to 10 min at 37°C to complete lysis.

8. Vortex at highest setting for 30 seconds in an appropriate tube (either a 1.5 ml eppendorf tube or a 15 ml tube).

9. Add 27.6 μl (2.4 ml) of fresh 3.0 N NaOH and mix gently by inverting in an eppendorf tub or by swirling in a 250 ml centrifuge bottle for a large scale prep.

10. Add 49.6 μl (3.9 ml) of 2.0 M Tris-hydrochloride, pH 7.0 and continue gentle mixing for 3 min.

11. Add 71.7 μl (5.7 ml) of 5.0 M NaCl .

12. Add 700 μl (55.8 ml) of phenol saturated with 3% NaCl and mix thoroughly.

13. Centrifuge for 5 minutes in a microfuge or at 5,000 rpm in a GSA rotor for 10 minutes.

14. Remove the upper phase and extract with 700 μl (55.8 ml) of chloroform-isoamyl alcohol (24:1).

15. Remove the upper phase and add 1 volume of isopropanol.

16. Incubate for 30 minutes at 0°C or for over 1 hour for a large scale prep.

17. Centrifuge for 5 minutes in a microfuge or at 8,000 rpm in a GSA rotor for 20 min.

18. Remove the excess isopropanol and resuspend in 20 μl (1,200 μl) of 10 mM Tris-1 mM EDTA, pH 7.5.

19. Examine 5 to 10 μl by agarose gel electrophoresis.

Agarose gel electrophoresis:

Perfrom agarose gel electrophoresis in a Tris acetate buffer containing 40 mM Tris, 20 mM acetic acid, and 2 mM Na_2 EDTA (pH 8.1). Gels should contain 0.6% agarose. Perform electrophoresis at 100V (3.6V/cm) for 5 h. Stain gels with 0.5 μg of ethidium bromide per ml and photograph through a red filter on Polaroid type 107 film.

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MITOCHONDRIAL DNA RESTRICTION ENDONUCLEASE PATTERNS IN SORGHUM

KEY WORDS: Mitochondria, Chromosome, Restriction endonuclease, Electrophoresis

BACKGROUND:

Three organelles in plant cells contain DNA (deoxyribonucleic acid), the primary genetic material in all living organisms. They are the nucleus, the mitochondrion, and the chloroplast. For most plant species, mitochondria and chloroplasts are maternally inherited, i.e., the female plants contribute mitochondria and chloroplasts to the offspring, and the male plants contribute only one-half the nuclear genetic material.

Mitochondria and chloroplasts have smaller genomes than that of the nucleus. Each mitochondrion contains 200 to 2,500 kilo base pairs (KBP) and chloroplast, 120 to 160 KBP, with very few exceptions. Unlike the nuclear genome, mitochondrion and chloroplast genomes are conservative and have much less repetitive DNA. The conservativeness and small size of mt- and cp-genomes provide the logical basis for evolutionary studies.

Mitochondrial and chloroplast genomes are very important components in agricultural production. Both are known to carry genes controlling cytoplasmic male sterility, an important trait in commercial hybrid production, such as hybrid corn and hybrid sorghum. Chloroplast genomes also are known to carry herbicide resistance genes, an important consideration for today's modern agriculture.

The genus *Sorghum* consists of a group morphologically diverse species and their somatic chromosome number falls into four categories: 2n = 10, 2n = 20, 2n = 30, and 2n = 40. The phylogenetic relationships among those species of various ploidy levels are not clear, except that *Sorghum bicolor* (2n = 20) could be one of the progenitors of *Sorghum halepense* (2n = 40) from chromosome pairing analyses in their 30-chromosome hybrids and 60-chromosome amphiploids. Sexual hybridization between 10-chromosome *Parasorghums* and 20-chromosome *Eusorghums* has never been successful. Histological studies on species hybridization reveal that most pollen grains of the 10-chromosome sorghums fail to germinate on the stigmatic surface of the 20-chromosome sorghums and vise versa. For those pollen grains that do germinate, pollen tubes grow so slowly that they never reach the ovules at the micropylar end.

With the advent of molecular biology and biotechnology, analysis of organelle genomes such as mitochondrial and chloroplast DNA in sorghum and other plant species can be made using restriction endonucleases which cleave at the DNA molecules only at well-defined restriction sites. Analyses of organellar genomes shall provide a better understanding on species interrelationships, the organization of those organelles, and the role they may play in agricultural production.

STATEMENT OF PROBLEM:

To construct and compare the restriction fragment length polymorphism (RFLP) of mitochondrial and chloroplast DNA of sorghum species at various ploidy levels and between male fertile and sterile stains.

METHODS AND MATERIALS:

Many plant taxa consist of species with a series of ploidy levels. Some examples are: *Triticum* (wheat) consists of hexaploid (*Triticum aestivum*, 2n = 6x = 42), tetraploid (*Triticum durum*, 2n = 4x = 28), and diploid (*Triticum monococcum*, 2n = 2x = 14), the genus *Nicotiana* (tobacco) consists of tetraploid (*Nicotiana tabacum*, 2n = 4x = 48) and diploids

(*Nicotiana sylvestris*, 2n = 2x = 24); the genus *Sorghum* (milo) consists of octoploid (*Sorghum halapense*, 2n = 8x = 40), tetraploid (*Sorghum bicolor*, 2n = 4x = 20), and diploid (*S. versicolor*, 2n = 2x = 10), and the genus *Solanum*, the ploidy levels and mutual relationship among species at different levels are known. Wheat and sorghum are monocotyledonous while tobacco and potato are dicotyledonous species. All those materials are easily accessible from many land-grant universities, such as Kansas State University.

Availability of cytoplasmic male sterile (cms) and its fertile counterparts have greatly facilitated the production of commercial hybrids. In corn (*Zea mays*), three types of cms strains are known: T, S, and C types each requiring a different restore nuclear gene to restore the fertility. In sorghum, only one major cms type is in use, the A_1 type, although other cms types are known. Cytoplasmic male sterility occurs in a wide range of plant species, including *Brassica* (cabbage), *Nicotiana* (tobacco), *Beta* (sugarbeets), *Medicago* (alfalfa), and many others. These cytoplasmic male steriles and their male-fertile maintainer and restorer lines also can be obtained from many land-grant universities.

The method that we have been using in extracting the mt- and cp-DNA from **sorghum seedlings** are described as following:

A. Isolation and purification of mitochondrial DNA

1. Sterilize 10 g of seeds from each source (cms strains or A lines, fertile strains or B lines, and/or fertility restore strains or R lines; or plants from various ploidy levels of a given species) with 50% commercial clorox (or 2.7% NaOCl) for 10 min with agitation (use a magnetic stirrer), rinse with tap water for 1 hr, submerge in 70% ethanol for 2 min (to break the surface tension and kill germs in crevices), rinse again with autoclaved sterile water.

2. Place the sterilized seeds in 150 x 20 mm sterile glass (or disposable) Petri dishes or use several 100 x 15 mm dishes containing two layers of sterile moist blotting paper (or paper towel). Seal the Petri dishes with parafilm and keep in darkness for a week at 24°C (use an incubator or an oven).

3. Collect the etiolated mesocotyl and coleoptile (the yellowish shoots above the seeds) by cutting with scissors, weigh 5 to 10 g of fresh tissue for homogenization.

4. Homogenize the etiolated seedlings for 2 min using a mortar and pestle (or a blender). For every g of fresh tissue, use 3 volumes of isolation buffer (0.5 M mannitol, 0.01 M N-Tris hydroxymethyl 1-2-aminoethane sulfonic acid (pH 7.2), 0.005 M EDTA (Na_2-ethylene diamine tetraacetic acid, pH 8.0), 0.15% BSA (Bovine serum albumin), and 0.05% cysteine.

5. Filter the homogenate through 4 layers of cheesecloth and 1 layer of miracloth, that have been presoaked with isolation buffer, into a 50 ml centrifuge tube.

6. Centrifuge the filtrate at 1,000 xg for 10 min using a centrifuge. Collect the supernatant and centrifuge again at 12,000 xg for 20 min to obtain crude mitchondrial pellet.

7. Resuspend the pellet gently in a 10 ml isolation buffer using a small paint brush and centrifuge again at 1,000 xg for 10 min. Collect the supernatant for DNase treatment.

8. Treat the supernatant with $MgCl_2$ and DNase at respective concentrations of 0.01 M and 10 μg/g fresh tissue in order to remove nuclear DNA contamination. The reaction time is 1 hr on ice and terminates the reaction immediately by subjecting the solution to sucrose gradient purification.

9. Purify the isolated mitochondria by layering the mitochondria on top of 20 ml washing buffer (0.6 M sucrose), 0.01 M TES (N-Tris (Hydroxylmethyl) methyl-1-aminoethanesulfonic acid; 2-((2-Hydroxyl-1,1-bis(hydroxymethyl)ethyl)amino) ethane sulfonic acid), pH 7.2), 0.02 M EDTA (pH 8.0) and centrifuge at 14,000 xg for 20 min. Resuspend the pellet in 10 ml washing buffer and centrifuge at 750 xg for 10 min. Collect the supernatant and centrifuge twice at 12,000 xg for 20 min. The pellet contains the purified mitochondria.

10. Resuspend the pellet in 2 ml of lysis buffer [0.05 M Tris-HCl, pH 9.0; 0.01 M EDTA, pH 8.0, 2% Sarkosyl, 0.02% proteinase K] and incubate at 37°C in a water bath with gentle shaking (40 rpm) for 1 hr.
11. Place the lysate in a 15 ml Kimax tube. Add equal volume of TRIS-saturated phenol pH 8.0, and mix gently for 2 min. Then add equal volume of chloroform-alcohol mixture (chloroform:isoamyl alcohol = 24:1) for 2 min. Centrifuge the Kimax tube at 1,900 xg for 10 min at 24°C. Transfer the upper aqueous phase to another Kimax tube and repeat the phenol/chloroform-alcohol extraction 3 times, which is necessary to purify the mt-DNA by deproteinization.

12. Add ammonium acetate to make it 0.5 M in concentration. Add 2 volumes of 100% ethanol to the final aqueous phase in a siliconized 15 ml Corex tube. Mix thoroughly but gently. Store the mixture at -20°C overnight.

13. Harvest the mt-DNA pellet by centrifuging at 12,000 xg at 4°C for 25 min. Wash the pellet by adding 1.4 ml 70% ethanol to the Corex tube and incubate at 37°C for 10 min.

14. Transfer the mt-DNA-ethanol mixture to a 1.5 ml Eppendorf microcentrifuge tube and centrifuge at 14,000 xg at 4°C for 25 min.

15. Remove the supernatant and dry the DNA pellet for 7 min. Resuspend the purified mt-DNA in 30 to 80 μl of 10 mM Tris-HCl (pH 8.0) and 5 mM EDTA (pH 8.0).

16. Further purification of mt-DNA by adding spermine to the DNA sample until a concentration of 100 mM is obtained. Mix the solution gently and keep it at 4°C for 15 min, and centrifuge at 14,000 xg at 4°C for 20 min. Remove the supernatant carefully. Add 10 ml spermine extract in buffer (75% ethanol, 0.3 M sodium acetate, and 0.01 M magnesium acetate) to the DNA pellet. Mix the solution gently once every 15 min at 4°C for 1 hr.

17. Centrifuge the solution at 14,000 xg for 20 min at 4°C; remove the supernatant. Vacuum dry the DNA pellet for 7 min and resuspend in the original volume of 10 mM Tris-HCl (pH 8.0) and 5 mM EDTA (pH 8.0).

B. Restriction endonuclease digestion

1. Use 2 or more restriction endonucleases (*Eco*R I, *Hin*d III, *Xho* I, *Bam* HI, *Pst* I, etc.) to digest the DNA. The supplier of the enzymes usually provides a recommended procedure. For example, the digestion condition of EcoR I is: 1 mM DTT (DL-Dithiothreitol), 2 mM spermidine, 100 mM NaCl, 50 mM Tris-HCl (pH 8.0), and 0.25 μg/μl BSA.

2. Digest 5 to 6 μg mt-DNA in 20 μl reaction mixture with 20 to 40 units of the restriction enzyme. Incubate at 37°C for 6 hr. The reaction is terminated by adding 5 μl loading dye (0.25 bromophenol blue, 0.25% xylene cyanol, 0.25% Ficoll [Type 400]). Store the digested mt-DNA immediately at -20°C.

C. Agarose gel electrophoresis

Fragments of the digested mt-DNA are separated by electrophoresis in 0.7% agarose horizontal slab gel at 3 v/cm for 17 hr at 22°C in TBE buffer (0.089 M Trisma base, 0.089 M Boric acid, and 0.002 M EDTA).

Stain the gel with 0.6 μg/ml Ethidium bromide for 20 min and destain for 20 min in deionized water. Place the gel on top of a UV transilluminator and photograph through Kodak Wratten No. 9 and No. 23 filters with Type 55 P/N Polaroid film.

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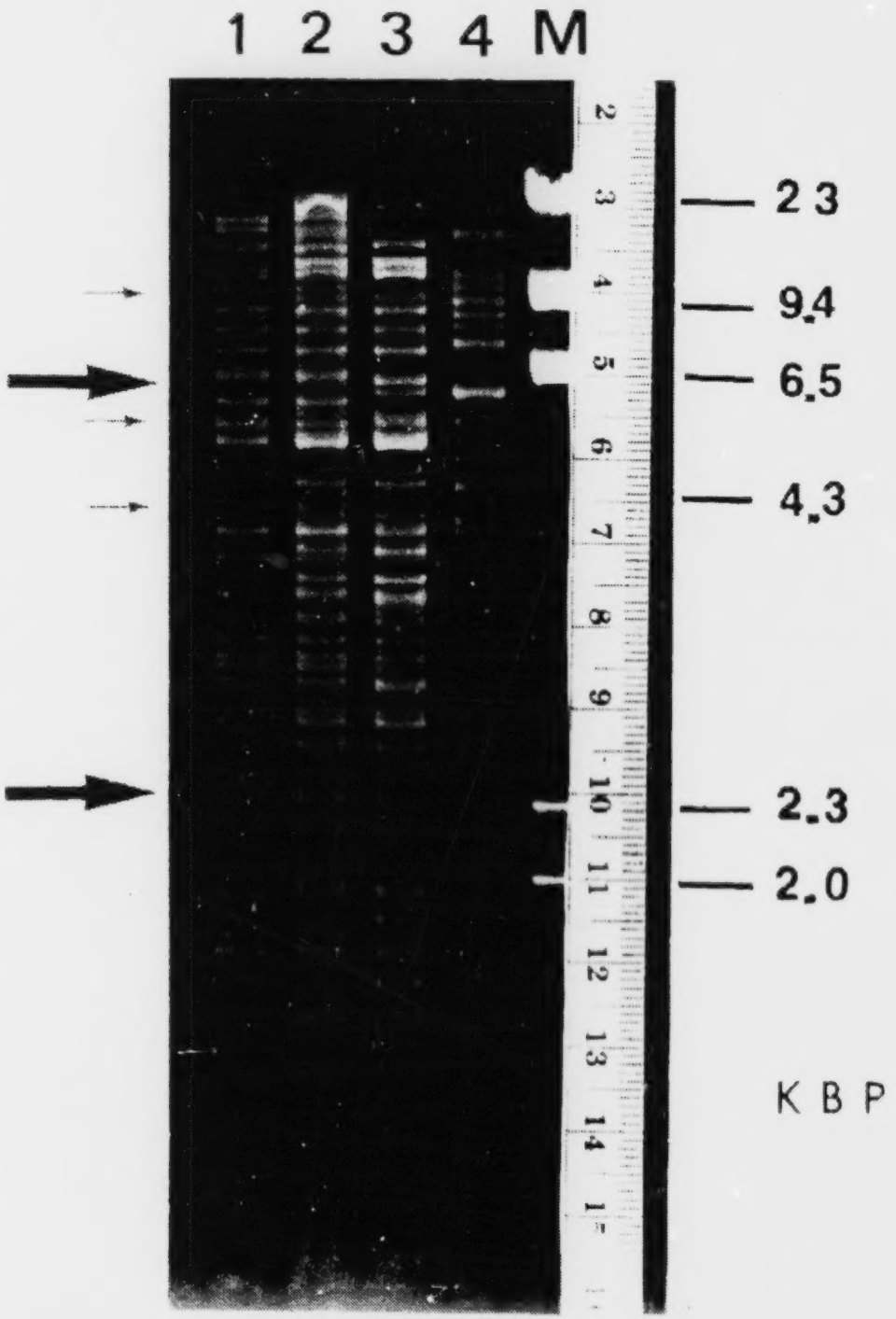

Restriction endonuclease patterns of EcoRI digestion of mt-DNA from sorghums. Lane 1, *S. halepense* (2n=40); Lane 2, Redlan B (*Sorghum bicolor* race bicolor, 2n=20 male fertile); Lane 3, Redlan A (male sterile counterpart of Redlan B); Lane 4, *S. versicolor* (2n=10) M=Molecular weight markers.

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RESTRICTION MAPPING OF INSECT BACULOVIRUS DNA

KEY WORDS: Baculovirus, Restriction Endonucleases, Electrophoresis, DNA.

BACKGROUND:

Biotechnology uses a number of methods for transport of DNA into various systems, thus allowing for expression of a gene in various cells or organisms. These vehicles for DNA transport are called vectors. One useful vector is the multi-encapsulated, double-stranded DNA nuclear polyhedrosis virus of the Cabbage looper, *Autographa californica* (AcMNPV). This vector has been used to produce about a dozen foreign proteins such as human ß-interferon. The reason this is such a valuable vector is that the promoter of the polyhedrin gene is very active, producing a large amount of proteins when activated.

Baculoviruses are large (80-150 kb) DNA viruses that infect insects, primarily lepidoptera. They occur as multiple virions embedded in a protein polyhedron or occlusion body (OB). Identification of these viruses has traditionally depended on the host from which the virus was isolated. With the advent of molecular techniques, identification can now be based on the restriction pattern of the viral DNA cut by restriction endonucleases and separated by agarose electrophoresis. These ladders of DNA are a means of establishing a physical map of the virus.

STATEMENT OF PROBLEM:

The problem is to determine if the Baculovirus genome changes when hosts are altered.

METHODS AND MATERIALS:

The following methods are used to isolate baculovirus DNA, restrict the DNA, and visualize the pattern by electrophoresis. This pattern can be used to compare different viruses, to compare the physical maps from the same viral strain raised in different hosts, and to determine from the physical maps whether the viral genome undergoes changes during alternate host infection.

Traditional methods of DNA isolation and visualization usually use ultra-centrifuges (those capable of exceeding a force of 30,000 xg), CsCl gradients and use of radioisotopes. The following are alternative methods that do not require these hazardous procedures.

Viral DNA isolation. The viruses are fed to first instar caterpillars and kept until the infection is in the last stages. The caterpillars can be bled and the OBs are visualized in the cells and hemolymph as refractal points when observed by a microscope. At this stage, caterpillars are placed in a beaker of distilled water (dH_2O) and allowed about one week to fall apart in the refrigerator (this takes about one week). The dense OBs fall to the bottom and can be collected in dH_2O. Cellular debris is removed by differential centrifugation (1000 rpm for 5 min).

The OBs are embedded in agarose beads. Take 2 ml OBs (2.4×10^9 OB/ml) and dilute to 5 ml with TE, pH 8/45°C, in a 125 ml flask. Add 5 ml 1% low melting agarose at 45°C and 20 ml light mineral oil at 45°C. Cover and swirl vigorously for 30 sec to get a uniform emulsion. Pour the emulsion into 100 ml ice cold TE, pH 8, stirring at medium speed in a beaker placed inside a second beaker packed with ice on a magnetic stirrer and stir 15 min. Centrifuge 1400 rpm for 10 min at RT. Remove the oil at the top, resuspend the pellet, recentrifuge, and remove excess TE. Resuspend with fresh TE, repeat centrifugation, and store pellet at 4°C with the addition of sodium azide to 0.01%.

The virions are freed from the OBs by treating the agarose pellets with an alkaline solution. Equilibrate the pellet in dH_20 on an orbital shaker at 50 rpm for 15 min. Remove the

dH_2O by centrifugation, repeat twice then add dH_2O to 20 ml. Add 20 ml dissolution buffer. Gently mix by inverting the tube for 5 min. Add 8 ml 2 mM NaH_2PO_4 to stop the dissolution by lowering the pH. Pellet the beads. Resuspend the beads in TE and wash 2X. Add 20 ml 4% SDS in 25 mM Na_2EDTA pH 8 and 0.3 M ßME (ßME has a strong odor and should be carefully handled with gloves). Shake horizontally 50 rpm for 30 min. Pellet the beads, remove the supernatant and add 20 ml of the 1% SDS in 25mM Na_2EDTA pH 8, 0.3 M ßME with 1 mg Proteinase K. Incubate overnight at 50°C in a waterbath. Pellet the beads and resuspend in 20 ml TE with 0.1 mM PMSF (freshly made) for 15 min at room temperature. Pellet and wash with TE three more times. Store at 4°C in TE. At this stage, the DNA has been isolated but is embedded in the agarose beads.

Restriction digestion. Place 20 µl of the slurry in a microfuge tube. Pellet the cells and suspend the pellet in 1X restriction buffer (supplied with the restriction enzyme). Incubate 5 min at room temperature and repeat twice. After the last wash, resuspend the slurry in 1X restriction buffer and add 1 µl restriction enzyme. Incubate at the appropriate temperature (usually 37°C) for 4 hrs. Pellet the beads and suspend in gel loading buffer.

Agarose gel electrophoresis. Load the sample into the well of a 0.7% agarose gel in TEB. Electrophorese the gel at 35V until the blue dye migrates to within 2 cm of the bottom. Stop the power, place in a pan with dH_2O and Ethidium bromide (Ethidium bromide is CARCINOGENIC and must be handled with gloves and carefully disposed of (see appendix)) and place on shaker table at low speed for 15 min. The EB is removed and washed with dH_2O for 15-20 min. The gel can now be photographed on a UV transilluminator. WEAR GLASSES that will prevent exposure to the UV. A pattern of DNA fragments can be detected and measured on the photograph. Procedures for photographing the gel can be found in Maniatis et al. (1982)

Chemicals and Solutions.

TE, Tris-EDTA (10 mM Tris, 1 mM Na_2EDTA, pH 8)

ßME, 2-mercaptoethanol (Sigma)

PMSF, Phenylmethylsulfonylflouride (Sigma) 200 mM stock in 100% 2 propanal
0.3484 g PMSF in 10 ml 2-propanol

Dissolution Buffer (40 ml) Na_2CO_3 (amhyrous) 3.39g 0.8 M
Sodium thioglycolate 0.02 M

TEB, Tris-EDTA-Borate (0.089 M Tris, 0.089 N boric acid, 0.002 M EDTA pH 8)

EB, Ethidium bromide (10 mg/ml, stock; 0.5 µg/ml, final concentration)

Additional projects. Other viral isolates can be examined and their restriction maps can be compared. Pulsed field electrophoresis can be used to resolve large DNA fragments in agarose gels. The fragments can be cut out of the gel and used to make probes with biotin-label for Southern hybridizations or to clone. These techniques require advanced expertise, and researchers at a university should be contacted for advice.

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SELECTED APPLICATIONS OF TECHNIQUES

SOUTHERN BLOTTING OF DNA

Southern blotting of DNA is a basic technique used in biotech labs. It is recommended that human blood or blood products *not* be used as a source of DNA by high school students.

KEY WORDS: DNA, Southern Blot, Restriction, Fragment, Electrophoresis, Probe

BACKGROUND:

The ability to detect a specific DNA sequence or gene in a large population of different DNA sequences is often critical to a successful DNA cloning or gene analysis experiment. The difficulty in doing the detection can be compared to "finding a needle in a hay stack". The Southern blot procedure, developed by and named for E.M. Southern in the early 1970's, is still the best method for detecting that one specific DNA sequence of interest.

In a typical Southern DNA detection procedure, DNA (for example, chromosomal or genomic DNA) is digested with a restriction endonuclease. Restriction endonuclease digestion can produce several thousand DNA fragments (restriction fragments) of different sizes and sequences. Only one of these is the restriction fragment that contains the gene of interest.

The DNA restriction fragments (the "target" DNA in the Southern blot procedure) are separated, according to their size, by agarose gel electrophoresis. The target DNA is denatured, transferred (blotted) and bound to a solid support, typically, a nitrocellulose membrane filter by the Southern blot procedure. A purified DNA fragment of known sequence (the "probe") that is complementary to the target DNA you want to find is used to "fish" for the target DNA on the nitrocellulose membrane filter. The probe DNA must be "tagged" with either a radioactive or non-radioactive label. The labeled probe DNA specificaliy binds (hybridizes) only to its complementary target DNA on the membrane filter. The specific hybridization of probe and target DNAs can be visually detected because of the label on the probe DNA.

Southern blotting has significantly helped the development of modern molecular biology. It has helped researchers clone rare genes and answer important questions on how genes and DNA expression are regulated in the cell. Recently, Southern blotting has had a dramatic impact on our understanding and diagnosis of human genetic diseases.

EXPERIMENT:

Perform a Southern blot of target DNA restriction fragments from an agarose gel to a nitrocellulose filter.

MATERIALS:

Several sophisticated Southern blot apparatuses are commercially available at a cost of $100 - $300. We describe here a simple Southern blot procedure using inexpensive and readily available reagents.

- Nitrocellulose membrane filter (available from Fisher Scientific): cut to the same dimensions as the agarose gel used to separate the DNA restriction fragments. Wear non-porous gloves when handling the nitrocellulose filter. Finger prints on the filter will interfere with detection of the DNA. The nitrocellulose filter is fragile. Handle it carefully.

- Blotting buffer: 0.4 N Sodium hydroxide (NaOH) Caution: NaOH is caustic and can cause skin burns. Wear non-porous gloves and eye protection when working with NaOH.

- Glass tray: 9 X 9 in or larger.
- Sponge: Kitchen sponge cut a little larger than the dimensions of the agarose gel.
- Whatman 3MM paper: 8 pieces cut to the size of the agarose gel.
- Paper towels: 3 inch stack cut to the size of the agarose gel.
- Weight block: 2-3 lb (1 Kg) lead or other metal block.
- Neutralization buffer : 0.3 M Sodium Chloride (NaCl), 40 mM Sodium citrate, pH 7.0.

PROCEDURE:

1. Run DNA restriction fragments on a 10 cm x 10 cm agarose gel as described in Molecular Cloning by Maniatis *et al*, 1982.
2. Add Blotting buffer to the glass tray to a height of approx. 2 cm. Soak the sponge in the tray. Caution: wear non-porous gloves. Lay the sponge in the center of the tray.
3. Center 4 pieces of Whatman 3MM paper on top of the sponge. Remove the agarose gel from the electrophoresis chamber and place it upside down (so that the gel slots are down) on the Whatman paper.
4. Carefully Pre-wet the nitrocellulose membrane filter in Blotting buffer. Carefully place the moistened filter onto the gel. Use a glass rod or 5 ml pipet and gently roll across the membrane filter. This is done to remove any air bubbles between the gel and filter and allow efficient transfer of the DNA from the gel to the nitrocellulose membrane filter.
5. Place 4 pieces of Whatman 3MM paper on top of the nitrocellulose membrane filter. Place 2-3 stack of pre-cut paper towels on top of the Whatman paper. Place the lead weight on top of the paper towel stack. After the lead weight is added, check to be sure that the second 4-piece stack of Whatman paper and the paper towels are not touching the Blotting buffer or the sponge.
6. Add Blotting buffer to the tray until it is within 2 mm of the top of the sponge. Allow the blotting process to go overnight. During the overnight blot, the DNA is transferred to the nitrocellulose membrane filter by capillary action. The NaOH Blotting buffer denatures the DNA as the DNA is being blotted onto the nitrocellulose.
7. Day 2: While wearing gloves, carefully remove the nitrocellulose membrane filter. Soak the filter in neutralization buffer for one minute. Bake the filter at 80°C for 1 hr. The baking step permanently binds the target DNA to the membrane filter.

The DNA restriction fragments have now been transferred and bound to the nitrocellulose membrane filter. The membrane filter can then be used for the detection of the single DNA restriction fragment of interest. The detection step will require the use of a DNA probe that has been radioactively or non-radioactively labeled. The Genius™ Nonradioactive DNA Labeling and Detection Kit from Boehringer Mannheim is an ideal kit to use for detection of DNA on Southern blots. Nonradioactive kits are also available from other companies. The kit provides restriction fragment target DNA controls, probe DNA, reagents for non-radioactive labeling of probe DNA, and reagents for detection of target DNA.

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DETECTION OF THE PHILADELPHIA (Ph') TRANSLOCATION USING A DNA PROBE TEST

This project demonstrates how the use of probes and Southern blotting can be used for a clinical application. Because of AIDS and other blood borne diseases, it is recommended that high school students not test for the Philadelphia chromosome. This procedure must be carried out in a clinical lab by individuals that are familiar with the proper techniques and precautions required for the handling of blood.

KEY WORDS: Luekemia, DNA, Southern blot, Probe.

BACKGROUND:

Chronic myeologenous leukemia (CML) is a pluripotent stem cell disorder which is characterized by the presence of a cytogenic abnormality termed the Philadelphia (Ph') chromosome. This abnormality is present in over 95% of patients with clinically diagnosed CML and results from a reciprocal translocation between the long arms of chromosome 9 and 22. During this event, the c-abl proto-oncogene (mapped to chromosome 9) is translocated to chromosome 22. In patients who exhibit the Ph' chromosome, the breakpoint on chromosome 22 maps within a gene designated "phl" and is localized within a 5.8 kb region of the phl gene, referred to as a breakpoint cluster region (bcr).

Methods for detection of the Ph' chromosome (translocation) are karyotype analysis and a DNA probe test.

STATEMENT OF PROBLEM:

Test if cancer patients have chronic myeologenous leukemia using a DNA probe test.

METHODS:

Protocols for DNA isolation, restriction enzyme digestion, gel electrophoresis, Southern blotting, 32P labeling and hybridization of Southern blots are available upon request from Oncogene Science.

EQUIPMENT:

Micro-tubes
Micro-centrifuge
Waterbaths
Gel electrophoresis equipment
UV light source
Blotting tray
Whatman 3MM paper
Nitrocellulose nylon filter
80°C oven
Geiger Counter
Liquid scintillation counter
Flat bottom (plastic) container
X-ray film
Intensifying screen
-70°C freezer
Liquid X-ray developer

MATERIALS:

OSI Transprobe kit (available from OSI)
- OSI Transprobe-1
- Ph' negative control DNA
- Ph' positive control DNA

Restriction analysis system (available from OSI or BRL)
- 1000 units Bgl II

- restriction enzyme buffer
- 10 μg control DNA
- sample loading buffer
- DNA size standards

Gel elctrophoresis
- electrophoresis grade agarose (BRL)
- electrophoresis buffer (90 mM Tris-HCl, pH 8.0, 90 mM H_3BO_3, 2.5 mM EDTA)
- ethidium bromide, 5mg/ml (potent carcinogen)

Probe labeling system (available from OSI, BRL and Pharmacia)
- 150 units DNA polymerase
- 500 μl random oligonucleotide primers
- 25 umoles of dATP, dGTP, dCTP and dTTP
- 32P-dCTP and 32P-dTTP (available from Amersham) Radioactive material; close supervision necessary

Hybridization and washing buffers (available from OSI and Oncor)
- Pre-hybridization buffer
- Hybridization buffer
- Wash solutions

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Editorial Note: 32P Labelling should be done with assistance from or by a scientist with expertice in handling 32P. Non radioactive methods, are also available.

PROTEIN ANALYSIS

MOLECULAR WEIGHT MEASUREMENTS OF SERUM PROTEINS

KEY WORDS: Electrophoresis, Blood, Serum, Protein.

BACKGROUND:

During many research experiments in the area of molecular biology, molecular weight measurements must be determined for unknown bio-molecules such as proteins, RNA and DNA. This is often accomplished using gel electrophoresis. This procedure takes advantage of the electrical charge on the surface of these molecules. To obtain a uniform electrical charge on protein molecules, they are first treated with a chemical (sodium dodecyl sulfate or SDS) to impart a uniform electrical charge on the molecular surface. DNA and RNA does not need to be treated with SDS due to the negatively charged phosphate ions on each nucleotides. These molecules are then placed in a gel matrix under an electrical field where negatively charged molecules will travel toward the positive electrode. The gel matrix acts as a sieve or filter in which the smallest molecules can travel more rapidly while the largest molecules travel very slowly. The relative migration of different molecules under the proper conditions will be directly proportional to the molecular weight of the molecule. Therefore, a series of molecules of known molecular weight can be run on a gel to generate a standard curve to which molecules of unknown size can be compared and their molecular weights measured.

STATEMENT OF PROBLEM:

Objective: To measure the molecular weights of unknown protein molecules.

METHODS AND MATERIALS:

This objective can be approached using sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS PAGE) following the procedure of Laemli using protein molecular weight standards. The unknown serum proteins can be obtained from a slaughter house, commerical sources, or insects (or skim milk could be used). Molecular weight standards for the gel can be purchased from a number of companies (i.e., Sigma Co., St Louis MO 63178, cat# MW-ND-500, [1-800-325-3010]). If there is no vertical gel electrophoresis system available, many biochemical laboratories at most colleges or universities, as well as pharmaceutical or industrial laboratories have these systems. These systems and their power supplies can be purchased from a number of companies (i.e., Bio-Rad Labs, Richmond CA. [1-800-645-3227], LKB-Pharmacia, Piscataway, NJ [1-800-922-0318] etc.) along with pre-cast gels to run on these systems. It is recommended that the money be spent on pre-cast gels in order to minimize the exposure to acrylamide. (Note: acrylamide in the powder and liquid form is a dangerous neurotoxin; however, once it is crosslinked into a gel it is no longer as dangerous.) Gloves should be worn at all times during the procedure.

Pre-cast Laemli gels with sample-well combs should be attached to the electrophoresis apparatus according to manufacturers procedures. The bottom (positive electrode) and top (negative electrode) buffer reservoirs should be filled with "Running Buffer" (see Solutions below) so that the bottom and top of the gel are covered by buffer (follow manufacturer's recommendations). Unknown serum sample is diluted 1 part serum with 49 parts water to a final protein concentration of approximately 1 mg/ml. This diluted sample is mixed with "Sample Buffer" (0.033 ml sample Sample Buffer + 0.067 ml Diluted Sample final volume=0.10 ml). The molecular weight standards are mixed with "Sample Buffer" to a concentration of approximately 1 mg/ml. One hundred μl or 0.10 ml of the sample and standard are placed in separate tubes covered with marbles (1.5 ml capped Eppendorf tubes may also be used) and placed in boiling water for 15 min. Each tube is swirled to insure the sample is well mixed. Then 0.05 ml of each sample is placed in separate sample wells on the gel (Note: the sample volume may vary based on the gel system used). The glycerine in the "Sample Buffer" make

the samples denser than water so they sink to the bottom of the well. Place the cover on the electrophoresis unit and turn on the power supply. Run as described by the manufacturer (for a standard 1.5 mm thick x 10 cm gel, approximately 25 mAmps/gel and 2 Watts/gel for 3-5 hrs). The gel is then carefully removed (with gloves) from the glass plates that it was cast and run in. Very carefully the gel is placed in 200 ml of the staining solution for 1-16 hrs (see Solutions below). The stain is poured down the sink and replaced with "Destain" (see Solutions below), with periodic changes of "Destain" every few hours until protein bands are clearly visible. The migration distance of the standards and the unknown bands can be measured from the top of the gel with a ruler. A standard curve can be generated by plotting the Log of the molecular weight of the standards (Y-axis) against the number of mm traveled in the gel (X-axis). This should be a relatively straight line. Then locate the distance that an unknown band traveled in the gel and draw a straight line across to the Log of its approximate molecular weight.

SOLUTIONS:

Running Buffer

Trizma-Base (Sigma)	9.09 gm
Glycine (Sigma)	43.26 gm
SDS [Sodium Dodecyl Sulfate] (Bio-Rad)	15 mls of a 20% solution
Distilled Water (Final Volume)	3000 mls

Sample Buffer

Beta Mercaptoethanol (Sigma)	0.3 mls Use in Fume Hood
20% SDS	0.6 mls
1M Tris-HCl pH 6.8	0.37 mls
Glycerin (Sigma)	0.4 mls
0.4% Bromophenol Blue (Bio-Rad)	0.146 mls
Water	0.176 mls

Stain

Coomasie Blue 250 (Bio-Rad)	0.75 gm
Methanol	375 mls
Water	322 mls

Mix for several hours and filter through filter paper, store in brown glass away from light. For use, mix 139.5 mls Stain with 10.5 mls Glacial Acetic Acid.

Destain

Water	3500 mls
Glacial Acetic Acid	300 mls
Methanol	200 mls

Note: Gloves and lab coats should be worn during this procedure. Fume hood should be used with Glacial Acetic Acid, Beta Mercaptoethanol and Methanol.

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IDENTIFICATION OF SEAFOOD SPECIES USING ISOELECTRIC FOCUSING TECHNIQUES

KEY WORDS: Protein, Isoelectric focusing, Electrophoresis, Species identification.

BACKGROUND:

There has been a tremendous growth in seafood consumption in this country due to changes in consumer attitudes toward health and nutrition. With the increased demands for higher quality seafood products, consumers may have already encountered the willful or unintentional adulteration by dealers who substitute higher quality and priced seafood with lower quality and less expensive products.

Identification of seafood species can be difficult. The use of morphological characteristics for identification requires a great deal of experience. In addition, the morphological characteristics can be lost when fish fillets are prepared or when the seafood is processed. Electrophoretic methods have been widely used for seafood species identification. By using isoelectric focusing (IEF), Lundstrom (1981, 1983) and Hamilton (1982) have identified successfully Pacific rock fish and other fish species such a cod, haddock, pollock, cusk, red hake, red snapper, weakfish, American plaice and gray sole. The pink, white and rock shrimp species can also be identified using the urea gel IEF technique (An *et al.*, 1989).

STATEMENT OF PROBLEM:

This study is designed to verify if the fish (or shrimp) species can be identified by comparing the muscle protein banding patterns, obtained by the urea gel isoelectric focusing technique, of the individual species with those of the reference database.

METHODS AND MATERIALS:

Fish species such as grouper, cod, flounder and red snapper, and shrimp species such as pink, white and rock shrimp, will be purchased from the marketplace. The chopped samples at 8 g will be mixed in separate beakers with 24 ml water (1:3 ratio, weight/volume) containing 0.1 mM phenylmethylsulfonyl fluoride (PMSF), 10 mM EDTA and 0.01% sodium azide. The mixed sample will then be homogenized at room temperature for 1 min using a Polytron (Brinkmann Instrument, Westbury, NJ) (setting 9.2). Alternatively, a blender and Dounce Tissue grinder may be used. The homogenates are then centrifuged at 26,900 x g for 20 min at 5°C. After the supernatants are collected and the protein concentration determined following the methods of Lowry *et al.* (1951), the supernatants are adjusted to 5 mg/ml with water. After 3-4 granules of sucrose are added to 0.1 ml of supernatant, 0.1 mg protein is applied to the IEF gel.

A gel mixture containing 4% acrylamide, 2% Triton X-100 and 9.2 M urea will be mixed for 5 min at 37°C. Following the addition of the pH 3-10 ampholyte at a final concentration of 6.2%, the gel mixture will be degassed for 5 min by appllying a vaccum to the flask used for mixing. After fresh ammonium persulfate solution and N,N,N',N'-tetramethylethylenediamine (TEMED) are added at the respective final concentration of 0.02% and 0.14%, the mixture will be poured into 16 x 20 cm slab gel plates (0.75 mm thick) assembled with a comb. The gel is then allowed to polymerize for 1 hr. Following the removal of the comb from the gel, lysis buffer containing 9.5 M urea, 2% Triton X-100 and 2% ampholyte will be overlayed on the gel. The gel is again allowed to sit for another hour. After prefocusing of the gel is done as previously described (An *et al.*, 1989), protein samples (0.1 mg/well) as well as the protein standards (Broad pI kit, pH 3-10, purchased from Pharmacia, Piscataway, NJ) are applied to the gel and overlayed with an aqueous solution containing 2% Triton X-100 and 2% ampholyte. The gel plate will then be reassembled in the electrophoresis unit, and after fresh cathode solution is added to the chamber, proteins will be focused and

stained with Coomassie blue R-250 as previously described (An *et al.*, 1989). The protein banding patterns of the samples will then be compared to those of the authentic standards, and the species origin of the samples will be identified.

Apparent pI values of the fish and shrimp proteins are determined indirectly by comparing their R_f values on the gel with those of the protein standards following the method of An *et al.* (1989). The pH profile of the whole gel will be determined at room temperature using a micro-surface pH electrode (Ingold Electrodes Inc., Wilmington, MA). A linear relationship of gel pH and the R_f values of protein standards enables determination of the apparent pI values for the corresponding protein standards.

Some of the chemicals used for this study can be toxic (sodium azide, acrylamide); they should be handled with caution. Students may gain access to a Polytron, centrifuge and an isoelectric focusing facility in the biology or biochemistry department at the universities. Since electricity at high voltages is used, students should be warned of the potential hazard.

Additional technical assistance can be obtained from the Bio-Rad (1414 Harbour Way South, Richmond, CA 94804) and Pharmacia LBK Biotechnology Inc. (800 Centennial Ave., Piscataway, NJ 08854).

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USE OF ELECTROPHORESIS FOR PLANT OR ANIMAL IDENTIFICATION

KEY WORDS: Electrophoresis, Protein.

BACKGROUND:

Much of modern biotechnology research involves the development of new strains of micro-organisms or new varieties of plants. In addition, new analytical and biochemical techniques are being developed to help distinguish between different naturally occurring varieties, subspecies, and species.

One particular problem of economic significance is the varietal identification of grain seeds and the testing of large batches of seeds for purity. Many states have official testing laboratories that test and certify seeds. For example, there are approximately 9,000 varieties of soybeans, and about 100 are commercially important. The differences between these varieties are very slight.

There is a growing interest in using electrophoretic techniques to rapidly identify plant or animal varieties. Electrophoresis, in general, is the separation of molecules (usually proteins or DNA) as they migrate through a solid medium while exposed to an electric potential. There are many different methods, depending on the solid media used. Cellulose acetate, agarose, starch gel, and polyacrylamide are the most frequently used materials.

The genetic information for each organism is contained in the DNA molecules. The more closely related two organisms are, the greater the similarity of the DNA. The sequence of bases on the DNA molecule carries the coded information that an organism needs to synthesize proteins.

Electrophoretic procedures for both DNA sequencing and protein separations are available. However, the information obtained by DNA electrophoresis is so extensive that it is much simpler to compare the protein electrophoresis pattern for organisms to help identify varieties. On a higher level, electrophoresis can also be used to identify proteins for different species (e.g., What kind of fish was really used to make that fish stick?).

STATEMENT OF PROBLEM:

First, find a situation where there is a need to rapidly identify a species or variety of plant or animal. Second, develop the appropriate electrophoresis procedure to prepare the sample and separate the proteins. Finally, compare the protein patterns of known samples to determine if this method can be used to identify unknowns.

METHODS AND MATERIALS:

A complete set of equipment for electrophoresis can cost anywhere from $200 to $3000. Many hospital labs and many college labs doing life science research may have systems that are not in use or have been replaced by newer models.

Some systems use up to 2,000 volts D.C., but many require just a few hundred. However, all standard equipment has built-in safety interlocks to protect the user.

What additional reagents and supplies will be needed will depend on the type of electrophoresis technique used.

Choose a method for conducting protein electrophoresis, develop the procedure so that you can differentiate species of fish. Then compare your system to another in order to determine if other systems work as well.

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THE MOLECULAR BASIS OF HETEROSIS: HYBRID ENZYMES IN HETEROZYGOTES

KEY WORDS: Crossbreeding, Complementation, Starch Electrophoresis, Drosophilia.

BACKGROUND:

Continuous inbreeding of a species produces inbreeding depression, which is a reduction in value for many traits including fitness and overall survival. Conversely, crossbreeding of two different inbreed strains will usually result in more vigorous hybrid offspring. This superiority is termed heterosis.

This observation has been heavily utilized in corn (*Zea mays*) breeding programs, since it has resulted in the trebling of average corn yields in the United States from the 1930's to the present.

There are two theories which have been proposed to explain this phenomenon. They are not mutually exclusive. The first, called the dominance hypothesis, states that inbreeding produces homozygosity for specific recessive deleterious genes and this reduces the overall fitness. The second explanation, called the overdominance hypothesis, depends on showing that heterozygosity of particular loci produces a phenotype superior to any homozygous state, regardless of the nature of the alleles at the locus.

Evidence for this second hypothesis includes the finding that random dimerization of polypeptides during synthesis in individuals heterozygous for two different alleles can produce new proteins with new and novel properties not found in the proteins produced by the homozygous strains (allelic complementation). In corn, Schwartz and Laughner showed that a hybrid has an alcohol dehydrogenase enzyme that is both active and stable under severe *in vitro* environmental conditions compared to inactive or unstable parental enzymes from homozygous plants.

STATEMENT OF PROBLEM:

To establish the degree of allelic complementation in enzyme systems resolvable by starch gel electrophoresis and histochemical staining.

METHODS AND MATERIALS:

Drosophila melanogaster is a suitable experimental organism. Various lines can be ordered from Carolina Biological Corporation or the Stock Center at Bowling Green State University. Inbreeding can be effected easily and generation times are short. Starch gel electrophoresis is easily done using glass plates and plastic strips to produce a three-dimensional mold. The strips can be cemented to the plates with non-silicon grease. Samples are loaded by squashing individual flies onto filter paper squares and inserting these into cuts made into the gel. A direct-current, variable-voltage power supply is needed. Relatively simple and inexpensive ones are available from biological supply companies. The gel is run by bridging the glass plate between two pyrex baking dishes containing suitable buffer and the current is allowed to flow through two filter paper bridges wetted with the same buffer. The entire assembly is covered with plastic wrap.

Many histochemical staining methods are available depending upon the enzymes studied. A comprehensive reference list is available in Harris and Hopkinson.

Inbreed flies for several generations; then outbreed and harvest the hybrid offspring. Compare one or more enzymes from the inbred versus hybrid flies. Look for the presence of new bands not found in the inbred stocks. Particularly look for staining ratios which may be

the result of random dimerization of monomers (or higher orders, see LDH). Treat the gels before or during staining with various physical factors including varying pH, temperature, substrate concentration, and possible enzyme inhibitors including soluble heavy metal ions. The degree of staining as compared to normal of the various isoenzyme bands may reflect increased sensitivity or stability of the enzymes. Different responses to the "heterozygote" bands compared to the "parental" bands would support allelic complementation and the overdominance hypothesis.

Caution must be exercised because of the electrical current needed to electrophorese as well as the poisonous nature of some of the reagents depending on the staining system.

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*Most university and college libraries will permit access to their journal collections for research needs.

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ISOLATION OF AN ABUNDANT PEROXIDASE ISOZYME FROM SOYBEAN

KEY WORDS: Peroxidase, Seed coat, Protein isolation, Chromatography column, Electrophoresis.

BACKGROUND:

In a broad sense, agricultural biotechnology involves the manipulation of crop plants so that they acquire new characteristics present in other living systems. Long-range goals are to introduce agronomically important traits such as an increased tolerance to environment stress. For example, such traits might include resistance to insect and microbial pests, drought, cold temperatures and high salinity. There is currently much interest in developing plants which are tolerant to herbicides. This would provide farmers a powerful method of weed control. The end result will be to increase the amount of food a farmer can produce per acre, which will become increasingly important as the world population density continues to grow. This technology also promises to provide the ability to increase the nutritional quality of the basic food supply by alteration of the amino acid composition of seed storage proteins.

The ability to transfer potentially beneficial traits from one organism to another requires the isolation and characterization of the gene or genes responsible for the trait, and the subsequent insertion of these genes into the genome of the plant species of interest. In most cases, the isolation of a gene requires the purification of the protein coded for by that gene. The amino acid sequence of the purified protein is determined, and a DNA probe can be synthesized which is complementary to the gene coding for that protein. The purified protein also allows one to generate antibodies which specifically recognize the protein of interest. Both of these tools, i.e., DNA probes and antibodies, can be utilized to isolate the gene of interest. Therefore, a working knowledge of protein chemistry and molecular biology are fundamental prerequisites for success in the general area of biotechnology.

The isolation of a protein which may comprise a very small percentage of the total protein present in a particular organism can be a formidable task. In order to accomplish the isolation of a protein, a basic understanding of conventional biochemical separation techniques and a knowledge of the molecular characteristics of the protein in question are necessary. The isolation of proteins is based on several molecular parameters such as the protein's size, electrical charge, and affinity for various biological molecules. This exercise is designed to introduce students to a few of the techniques employed in the isolation and analysis of proteins.

STATEMENT OF PROBLEM:

The objective of this experiment is to purify an abundant enzyme (protein) present in the protective covering or coat of soybean seeds.

METHODS AND MATERIALS:

This protein is stable at room temperature and the assay for its activity is relatively simple. Moreover, the experiment can be accomplished in a short period of time (2-3 days), and there are several convenient points during the purification where the experiment can be interrupted and the sample stored until the researcher is able to resume experimentation. The experiment is an ideal research project for students interested in protein purification, yet are unfamiliar with many of the problems that can be encountered during the isolation of a protein.

Isolation of Soybean Seed Coats:

Post-harvest soybean seeds can be obtained from any local seed distributor (seeds are most available in the spring months prior to planting). A typical experiment requires 10-25 g

of seed coats which are obtained from 200-500 g of soybean seeds. The seed coats are removed from the seed by placing 10-20 seeds in a petri dish containing distilled water, allowing them to imbibe water for approximately 5 min or until the coat starts to become wrinkled. The coat is removed most efficiently by peeling the coat away from the seed. This process is repeated until a sufficient quantity of coats is obtained. The coats should then be stored in a freezer until the researcher wishes to prepare a coat extract.

Preparation of Coat Extract:

Coats are placed in a flask or beaker containing 25 mM KH_2PO_4 (potassium phosphate) buffer, pH 7.5, at a ratio of 10 g of wet coats per 100 ml buffer. Homogenize the coats with a Tissuemizer at a speed which does not cause the sample to foam (foaming denatures proteins in the solution and may result in a loss of activity). Alternatively, dry seed coats can be frozen with liquid nitrogen and ground to a fine powder using a mortar and pestle. The powder is then stirred in an appropriate amount of buffer for one hour. Filter the homogenate through three layers of cheesecloth to remove large cellular debris. Transfer the homogenate to a centrifuge tube and centrifuge for 10 min at approximately 10,000 rpm at 4°C. Carefully remove the supernatant fluid by pouring it into a new beaker. This material can be stored below 0°C until the supernatant fluid can be fractionated on DEAE-Cellulose.

Fractionation on Diethylaminoethyl (DEAE)-Cellulose:

This technique takes advantage of the fact that the protein of interest contains an overall net negative charge at pH 7.5. The charge is due to the presence of acidic amino acid residues located on the surface of the protein. DEAE-Cellulose is composed of positively charged (basic) functional groups covalently linked to an insoluble matrix (cellulose). When the protein is applied to the DEAE-Cellulose, the attraction between the negatively charged surface groups on the protein and the positively charged groups of the DEAE-Cellulose causes the protein to bind through ionic interactions. The protein can then be eluted or released from the insoluble matrix by increasing the salt (KCl) concentration. When salt is present, the negatively charged chloride ion associates with the positive charge on the DEAE-Cellulose and prevents the ionic interaction between the protein and the insoluble matrix. Negatively charged proteins have a tendency to bind to this matrix based on the strength of the ionic interaction. Some proteins have a small tendency to form an ionic interaction with the matrix, while others have a higher affinity for the matrix. If a gradient of salt is applied to the matrix, the salt concentration can be increased gradually. Those proteins which are bound to the insoluble matrix weakly will be the first proteins to be released from the column, whereas the proteins strongly interacting with the matrix will require the presence of additional salt before they are released. This technique, ion exchange chromatography, can serve as a powerful purification method in that different classes of proteins can be separated from each other based on their overall net surface charge.

Apply the supernatant from the previous step to a DEAE-Cellulose column (2.5 x 20 cm) that has been equilibrated with 25 mM KH_2PO_4, pH 7.5. Equilibration is complete when the buffer flowing the column is the same pH as that which is applied. The proteins which do not have an affinity for the matrix will not interact and therefore flow through the column. This material should be checked or assayed to determine if the peroxidase activity has bound to the column. A simple assay procedure is accomplished by the addition of an equal volume of 2x activity stain to a small volume (25 μl) of the material to be analyzed. The presence of peroxidase activity will result in a rapid color change. The activity stain contains a colorless compound which, when oxidized by the enzyme, results in the appearance of a dark blue color. If the column is working properly, the peroxidase will bind and one should not observe a rapid color reaction in the material which does not bind to the column (flow through material). An assay for the protein of interest is a prerequisite to its isolation. The assay should be performed at each step during a purification procedure. It enables a researcher to monitor where the protein is during the course of the purification procedure. After the sample has passed through the column, an additional column volume of 25 mM KH_2PO_4, pH 7.5, should be added to the

top of column and allowed to flow through (this is known as the wash step) and serves to remove any unbound proteins from the matrix. At this point the upper portion of the column should be a rust brown color due to the presence of the bound peroxidase. The color is present because the peroxidase contains an iron-containing cofactor (heme) very similar to the heme group found in hemoglobin. The peroxidase can be eluted from the column by the addition of a linear gradient (0.025-0.5 M) of KCl. Linear salt gradients are formed by the gradual mixing of a solution containing high salt into a solution containing little or no salt. Once the salt gradient is applied to the column, a fraction collector containing test tubes must be attached to collect individual fractions of material as it elutes from the column. The gradient is formed by adding 50 mls of 25 mM KH_2PO_4, pH 7.5, in the gradient former chamber which contains the outlet that runs directly to the column. The second chamber is filled with 50 mls of 500 mM KCl, 25 mM KH_2PO_4, pH 7.5. To run the gradient, attach the gradient maker to the column by using a small piece of tubing, start the stirring apparatus, place the gradient former above the fraction collector and then open the channel between the two chambers. Upon completion of the gradient, assay a small portion of each fraction for the presence of peroxidase activity as described above. Those fractions containing peroxidase will be obvious due to the presence of the rust brown color. If a UV/VIS spectrophotometer is available, determine the optical density (O.D.) of the samples in each collection tube at 403 nm (the presence of heme) and at 280 nm (the presence of protein). One can determine the relative purity of this enzyme by dividing the O.D. at 403 by the O.D. at 280. The collection tube(s) containing the highest 403/280 ratio should be pooled and used for further purification on Con-A Sepharose. It is possible to stop at this point if it is not convenient to continue. If this is the case, the sample should be stored below 0°C.

Fractionation of Concanavalin A (Con A) - Sepharose:

The peroxidase of interest has been shown, by chemical methods, to be a glycoprotein (a protein which has covalently linked sugars on its surface). There are proteins, termed lectins, which have the ability to bind to sugar moieties. Concanavalin A (Con A) is one lectin which has been adapted for use in the purification of glycoproteins. This protein has been attached to an insoluble matrix (Sepharose) without severely altering its ability to bind sugars. Lectins have a high binding capacity or affinity for glycoproteins. A technique which is based on the affinity of one molecule (Con A) for another (glycoproteins) is called affinity chromatography. Proteins lacking sugar moieties will not bind to Con A Sepharose, thereby providing an efficient method of separating glycoproteins from nonglycosylated proteins. The glycoproteins that do bind to Con A can be further fractionated based on the affinity of the lectin for the glycoprotein. Separation of the bound glycoproteins is accomplished by gradually increasing the concentration of sugar much like the addition of increasing amounts of salt in ion exchange chromotagraphy. A linear gradient of increasing sugar results in the fractionation of proteins loosely associated with the lectin from those that are more tightly bound to the lectin.

The volume of the sample from DEAE-Cellulose is determined and 1/4 volume of 5x Con A-Sepharose binding buffer (250 mM Tris-HCl, pH 6.8, 2.5 M NaCl, 5 mM $MnCl_2$, 5 mM $CaCl_2$) is added. This solution is applied to a Con A Sepharose column (1.5 x 10 cm^2) which has been previously equilibrated with 1x Con A Sepharose binding buffer (dilute 1 part 5x binding buffer with 4 parts H_2O). It should be noted that the buffer present in the DEAE-Cellulose sample will not interfere with peroxidase binding to Con A Sepharose. The material which flows through the column during the application should be monitored for enzyme activity as described in the previous section. The upper region of the column should appear rust brown as it did on the DEAE-Cellulose column. The column should then be washed with one column volume of 1x Con A Sepharose binding buffer. A linear gradient of increasing sugar is applied to the column by the addition of 50 mls of 50 mM Tris-HCl, pH 6.8, 2.5 M NaCl to the outlet chamber of the gradient former and 50 mls of 50 mM Tris-HCl, pH 6.8, 2.5 M NaCl, and 1 M methyl-alpha-D-mannopyranoside (sugar) to the second chamber. The gradient is initiated as discussed above, and the peroxidase activity of the fractions eluting

from the column determined. The 403/280 ratio should be calculated for each tube collected. Combine only those tubes with intense rust brown coloring. These tubes should also display the highest 403/280 readings. Dialyze the sample against several 2 to 4 L changes of deionized H_2O to remove the copious amounts of salt and sugar. Transfer the contents of the dialysis bag to a clean container and freeze until the purity of the sample can be verified by polyacrylamide gel electrophoresis.

Preparation of SDS Polyacrylamide Gel:

The purity of any protein can be determined by SDS Polyacrylamide gel electrophoresis (SDS PAGE). This technique separates proteins on the basis of their molecular mass, hence the number of amino acids in a protein. The separation is accomplished by heating samples to be analyzed in a solution containing SDS (a negatively charged detergent) and dithiothreitol (a reducing agent). This treatment serves to coat the protein with detergent and to linearize the polypeptide. This technique allows a researcher to determine how many proteins are present in a sample of interest.

Electrophoresis is carried out in a vertical gel which is poured between two glass plates. There are two phases in the gel, the upper is the stacking gel and the lower is the resolving gel. The stacking gel has a low percentage of polyacrylamide and allows proteins to migrate without significant separation occurring. It is called a stacking gel because it allows the proteins to stack on the second phase, the resolving gel. The resolving gel, as the name indicates, resolves or separates the individual proteins. One must be careful in preparation in the materials involved in the technique because many of the reagents are potentially dangerous. Gloves should be worn at all times while handling these materials to prevent any contact. This technique should be performed with the assistance of an individual experienced in setting up an electrophoresis apparatus. Prepare the resolving gel by mixing the following reagents and/or stock solutions in the order of their appearance: 10 mls Acrylamide/Bis-acrylamide, 7.5 mls 1.5 M Tris-HCl, pH 8.8, 12.5 mls H_2O, 0.6 mls 10% SDS, 0.015 mls TEMED, and 0.2 mls 10% ammonium persulfate. This solution must be poured immediately between the two sealed electrophoresis plates because polymerization begins upon the addition of the final reagent. Gently layer a small amount of H_2O over the surface of the resolving gel to provide a smooth surface. Polymerization should be complete within 30 min. After the resolving gel has polymerized, remove the water from the surface and prepare the stacking gel mixture by mixing the following reagents in the order of appearance: 5 mls acrylamide/Bis-acrylamide, 7.5 mls 0.5 M Tris-HCl, pH 6.8, and 17.1 mls deionized H_2O, 0.3 mls 10% SDS, 0.015 mls TEMED, and 0.2 mls 10% ammonium persulfate. Pour this solution onto the resolving gel to the top of the glass plates. At this time, place the comb in the stacking gel and allow polymerization to occur. Attach the gel to the gel box and fill both chambers with running buffer. The running buffer is prepared by mixing 3.0 g Tris-HCl and 14.4 g glycine and diluting to 950 mls. Ten mls of 10% SDS is then added to the solution before diluting to 1000 mls. At this point, carefully remove the comb from the gel and clean the wells by gently pipeting running buffer in the wells. Attach the leads from a power supply so that the positive electrode is placed on the bottom buffer reservoir and the negative lead is attached to the upper buffer chamber. The gel should be allowed to pre-electrophorese at 150 volts for 2-3 hours prior to loading the gel. The protein samples, when placed in the wells, will migrate to the bottom of the gel because they all have a negative charge due to the presence of the SDS. Samples should contain approximately equal amount of protein. The protein concentration of the sample can be determined using a Bio-Rad protein determination kit (instructions included). Typically, 10 μg of protein from each sample are analyzed by this method. Add an equal volume of 2x loading buffer to the sample and boil for 5 min prior to sample application. After loading the gel, reconnect the power supply and electrophorese until the blue dye reaches the bottom of the gel.

Proteins may be visualized on an SDS polyacrylamide gel by several methods such as silver staining or staining with Brilliant Blue R (Coomassie Brilliant blue). Brilliant Blue R is less sensitive than silver staining, but the ease with which it can be performed outweighs the loss in sensitivity in this particular case. Once the blue dye has reached the bottom of the gel,

the current should be disconnected. The gel can then be transferred to a sufficiently large casserole dish containing Coomassie Brilliant Blue R staining solution. After one hour of gentle shaking, the staining solution is removed, then rinse the gel with water, and add 200 mls of Coomassie destaining solution. The gel is gently shaken in the destaining solution to facilitate the removal of the dye from the gel. The dye remains tightly bound to the protein during this washing such that protein bands will become visible as destaining of the gel proceeds. Several changes of the destaining solution (when the solution becomes blue) will decrease the length of time required for complete destaining. After the gel has been completely destained, any visible blue bands represent individual polypeptides (proteins) in the sample. Compare the number of polypeptides present in samples representing early stages of purification to those of the Con-A Sepharose elution sample. It should be obvious that each purification step has resulted in the removal of contaminating proteins. The intensely staining protein of the final purification step represents the major peroxidase in the soybean seed coat, and if the purification is done correctly only one band should be present in the Con-A elution sample. The molecular weight of the peroxidase is approximately 43 kD. This can be determined if molecular weight standards are also run on the gel. This preparation can now be used to generate antibodies or for the determination of the amino acid sequence, which can result in cloning of the gene encoding this protein.

The following list represents the equipment and supplies necessary for purification of the enzyme. We strongly suggest that this experiment be performed in a laboratory currently involved in protein purification, as the initial cost of performing such an experiment would be prohibitory if most of the equipment and reagents are not currently present in your laboratory. Interested students are more than welcome to perform this or related experiments in our laboratory. We can be contacted by mail at the address below or by telephone (419-372-8923).

Equipment:

Beckman High Speed Centrifuge J2-21
Beckman JA-20 Rotor
Centrifuge tubes with caps (Fisher Scientific 05-559-1E)
Cheesecloth (Fisher Scientific 06-665-25A)
Culture tubes, 13 x 100 mm (Fisher Scientific 14-961-28)
Econo-Column, 1.5 x 10 cm (Bio-Rad 737-1510)
Econo-Column, 2.5 x 20 cm (Bio-Rad 737-2520)
Fraction Collector (May be purchased through Fisher Scientific)
Gradient Former, Model 385, (Bio-Rad 165-2000)
Tekmar Tissuemizer or Mortar and Pestle
Whatman 1 Filter paper (Fisher Scientific 09-805F)
Vertical slab gel electrophoresis units and power supplies can be purchased from various sources such as Bio-Rad and Fisher.

Reagents:

Acetic Acid, Glacial (Fisher Scientific A38-500)*
Acrylamide (Fisher Scientific BP170-100)*
Ammonium Persulfate (Fisher Scientific BP179-25)
Bio-Rad Protein Assay Kit I (Bio-Rad 500-0001)
Bis-Acrylamide (Fisher Scientific BP171-100)*
Brilliant Blue R (Sigma Chemical Co. B 0630)
Bromphenol Blue Sodium Salt (Sigma Chemical Co. B 6131)
Calcium Chloride, ($CaCl_2$), (Sigma Chemical Co. C 3881)
4-Chloronapthol (Sigma Chemical Co. D 8890)*
Con-A Sepharose (Fisher Scientific 06-652-5A)
DEAE-Cellulose (Sigma Chemical Co. D 8507)
Dithiothreitol (Fisher Scientific BP172-5)

Glycine (Fisher Scientific G46-500)
Hydrochloric Acid, (HCl), (Fisher Scientific A144-500)*
Hydrogen Peroxide 30% solution, (H_2O_2), (Fisher Scientific H325-100)*
Lauryl Sulphate, Sodium Salt, (SDS), (Sigma Chemical Co. L 4390)*
Manganese Chloride, ($MnCl_2$), (Sigma Chemical Co. M 3634)
Methanol (Fisher Scientific A412-500)*
Methyl-alpha-D-mannopyranoside (Sigma Chemical Co. M 6882)
Molecular Weight Markers, 14-70 kD (Sigma Chemical Co. MW-SDS-70L)
Phosphoric Acid (Fisher Scientific A242-500)*
Potassium Chloride, (KCl), (Sigma Chemical Co. P 4504)
Potassium Hydroxide, (KOH), (Sigma Chemical Co. P 1767)*
Potassium Phosphate Monobasic, (KH_2PO_4), (Sigma Chemical Co. P 5379)
Sodium Chloride, (NaCl), (Sigma Chemical Co. S 9625)
Sodium Hydroxide, (NaOH), (Sigma Chemical Co. S 5881)*
Sodium Phosphate Dibasic, (Na_2HPO_4), (Sigma Chemical Co. S 0751)
Sucrose (Fisher Scientific S5-500)
N,N,N',N'-Tetramethylethylenediamine, (TEMED), (Sigma Chemical Co. T7024)*
Tris (hydroxymethyl) aminomethane hydrochloride, (Tris-HCl), (Fisher ScientificBP153-500)
*These chemicals have been shown to be hazardous.

Preparation of reagents:

Phosphate Buffered Saline, pH 7.2 (PBS)

200 mM Na_2HPO_4
1.5 M NaCl
Adjust the pH of the solution to 7.2

4-Chloronapthol solution

Dissolve 0.3g 4-chloronapthol in 100 mls of methanol

Activity Stain 2x

200 μl 10x PBS
400 μl 3 mg/ml 4-chloronapthol
398.5 μl H_2O
1.5 μl H_2O_2

Acrylamide/Bis-Acrylamide

Dissolve 30 g acrylamide and 0.8 g Bis-Acrylamide and dilute up to 100 mls. Filter this solution through Whatman No. 1 filter paper and store in the refrigerator. (Note: gloves and a mask should be worn when working with acrylamide)

2x Loading Buffer

0.2% SDS
10 mM Dithiothreitol (A fume should be used when working with this chemical)
10-15% Sucrose
0.002% Bromphenol Blue

Coomassie Stain

0.1 g Brilliant Blue R
75 mls H_2O
15 mls Methanol
10 mls Acetic Acid

Coomassie Destain

75 mls Glacial Acetic Acid
250 mls Methanol
675 mls H_2O

REFERENCES AND RESOURCES:

A public library contains inadequate literature pertaining to protein isolation methods; however, any college library should contain books which discuss protein purification methods. Any additional information pertaining to this study can be obtained from us at your request.

Schneiderman, H. A. 1987. What Biotechnology has in Store for Us. *The Ohio Journal of* Science Vol. 87(5):182-185.

Scopes, R. K. 1987. *Protein Purification: Principles and Practice*, Ed. C.R. Cantor, 1987, Springer-Verlag.

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INDUSTRIAL AND ENVIRONMENTAL BIOTECHNOLOGY

DETERMINATION OF ANAEROBIC BIODEGRADABILITY OF TOXIC COMPOUNDS IN WASTEWATERS

KEY WORDS: Wastewater, Biodegradation, Methane Potential.

BACKGROUND:

Wastewaters need to be treated to minimize pollution. One of the economic ways of treating wastewaters is biological treatment. Depending on the type of bacteria, the process could be aerobic or anaerobic. Anaerobic bacteria do not need oxygen, which reduces the cost of treatment as compared to the aerobic process. Anaerobic treatment has several other advantages. A useful end product of the anaerobic process is methane gas.

Toxic substances (organic and inorganic) may be present in wastewaters. The source of these toxic substances could be the industries in the area. It is required to know how these substances adversely affect the treatment process. Regulatory agencies like the Federal or State Environmental Protection Agency (EPA) are involved in determining whether the toxic organic compounds are aerobically or anaerobically biodegradable. During the past decade, a significant amount of work has been done by researchers in this area.

Measurement of Biochemical Methane Potential using serum bottles (small 100 mL bottles with rubber stoppers) is a standard technique for determination of anaerobic biodegradability of a wastewater. This technique will be used in this project (see Methods and Materials).

STATEMENT OF PROBLEM:

The objective of this project is to determine the *anaerobic biodegradability* of an industrial wastewater or a synthetic toxic wastewater. *Anaerobic biodegradability* of a wastewater means the potential for conversion of the wastewater to methane gas and carbon dioxide.

METHODS AND MATERIALS:

Serum bottles will be used to determine the biochemical methane potential (BMP) of the selected wastewater. The materials required are as follows:

1. Six Serum Bottles (100 to 150 mL each).
2. Rubber stoppers for the serum bottles.
3. A few 20 and 30 mL syringes.
4. Needles for the syringes.
   (see Fisher or other catalogs for items 1-4)
5. Collect 1-liter fresh anaerobically digested sludge for sewage treatment plant.
   (Gloves should be worn when working with the sludges).
6. Collect about 1 Liter of a selected wastewater or prepare a synthetic wastewater (e.g. 500 mg/L of glucose and 100 mg/L of formaldehyde or other chemical such as phenols).
7. Gas Measurement Apparatus. See Figure 1.

The following steps should be followed to perform the study:

1. Wash the serum bottles. Keep them upside down to drain the water. Label the bottles A-F.

2. Add 20 mL of digested sludge to each bottle.

3. Add 30 mL of the wastewater (with syringes) to bottles A, B and C. Add 30 mL of tap water to bottles D, E, and F (controls).

4. Put the rubber stoppers on the bottles.

5. Measure gas production daily (Mon - Fri). See Figure 1. of gas measuring apparatus.

6. Record gas data in a notebook.

7. Calculate the daily cumulative gas production.

ANALYSIS OF DATA:

After two weeks, plot the cumulative gas production versus time. If biodegradation occurs, the cumulative gas production in the test bottles (A, B, and C) will be higher than that of the control bottles (D, E, and F).

REFERENCES:

Castaldi, Frank J. 1981. Thermophilic Anaerobic degradation of phenobics. EPA 600/57 - 85.

Chakrabarty, A.M. 1982. Biodegradation and detoxification of environmental pollutants. LRC Press, Boca Raton.

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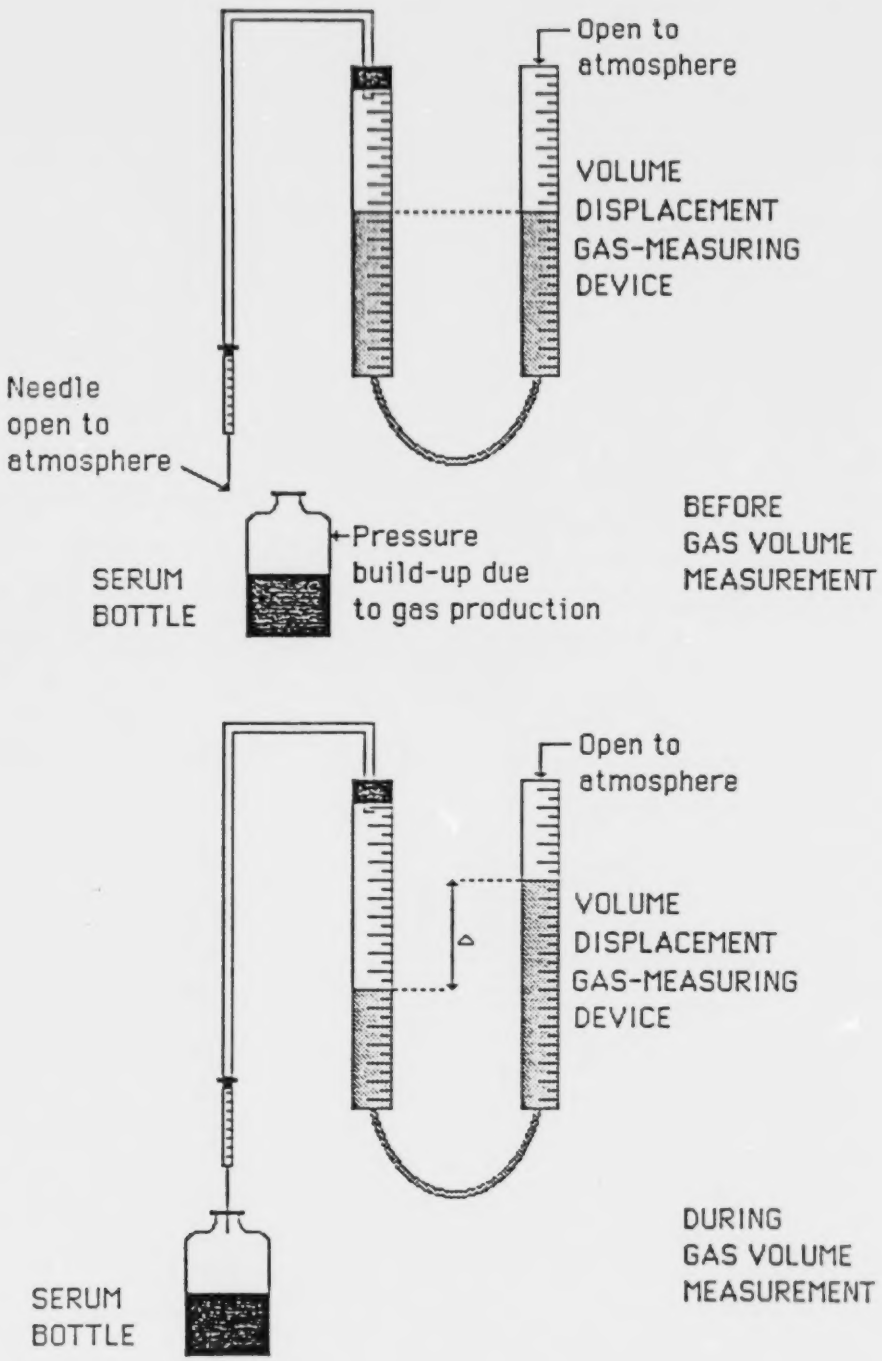


Figure 1. Anaerobic serum bottle gas measurement apparatus.

GROWTH OF A BACTERIAL ISOLATE ON THE BRANCHED HYDROCARBON LINALOOL

KEY WORDS: Pollution, Degradation, Linalool, *Pseudomonas fluorescens*.

BACKGROUND:

Bacterial isolates have been previously described that are able to utilize a wide variety of hydrocarbons and chlorinated hydrocarbons. Many of these chemical compounds are difficult to degrade in nature, and severe pollution problems can develop. One example would be the Love Canal area of New York. Currently, many agencies and companies in the United States are utilizing bacterial inoculants to degrade toxic compounds into harmless derivatives such as carbon dioxide and water. Bacteria such as *Pseudomonas fluorescens* represent useful alternatives to prolonged storage-burial and incineration of toxic hydrocarbons.

STATEMENT OF PROBLEM:

To investigate the growth of *Pseudomonas fluorescens* on linalool.

MATERIALS:

Glassware: Sterile disposable petri dishes.
Sterile disposable pipets.

Nephelo culture flask triple baffled, #2581
(Bellco Glass Inc. 1-800-257-7043).

One Pyrex brand desiccator (must be large enough to place several open petri dishes inside).

Equipment: One Spectronic 20 spectrophotometer
One flask shaker

Chemicals/media: MMO broth
465 ml of sterile distilled water
20 ml of sterile Na/K phosphate buffer pH 6.8
10 ml of sterile H-44 salts
5 ml of sterile 10% $(NH_4)_2SO_4$ solution

Sterilize all solutions separately and add to sterilized 465 ml distilled water.

MMO agar
465 ml of MMO broth supplemented with 10 g of Noble Agar (Difco).

Add 10 g of Noble Agar to 465 ml of distilled water and sterilize. After tempering at 45°C, add the stock solutions for MMO broth. Mix and pour into sterile petri dishes.

Stock Solutions for MMO.

Soln A: 1M Na/K PO_4 (pH 6.8)

1M Na_2HPO_4	141.96g/1000 ml
1M KH_2PO_4	136.09g/1000 ml

Mix basic to acidic for a final pH of 6.8 (1M Na/K PO_4)

Soln B: H-44 Salts (Hunters Vitamin Free Mineral Base)

Nitrilotriacetic acid	10.0 g (dissolve by adding 7.4 g of KOH)
$CaCl_2$-$2H_2O$	3.3 g
$MgSO_4$	14.45 g ($MgSO_4$ $7H_2O$ 29.59 g)
$(NH_4)_6Mo_7O_{24}\cdot 4H_2O$)	9.25 mg
$FeSO_4\cdot 7H_2O$	99 mg
Metal 44	50 ml
Final volume	1 Liter

Metal "44"

EDTA	2.5 (Na_2EDTA 3.2 g)
$ZNSO_4\cdot 7H_2O$	1.095 mg (dissolve 1.095g/100 ml water and add 0.1 ml)
$FeSO_4\cdot 7H_2O$	5.0 g
$MnSO_4\cdot 7H_2O$	1.54 g
$CuSO_4\cdot 5H_2O$	0.392 g
$Co(SO_3)\cdot 6H_2O$	0.248 g
$Na_2B_4O_7\cdot 10H_2O$	0.177 mg (dissolve 1.77 g/100 ml water: 1:100 dilution and add 1 ml to solution)
H_2SO_4	Add a few drops to retard ppt.

Soln C: 10% Ammonium sulfate

$(NH_4)_2SO_4$	100 g/1000 ml

Linalool 100 ml available through Aldrich Chemical catalog number L260-2 (1-800-558-9160).

Strain: *Pseudomonas fluorescens* PFL 7.0 (NRRL B-18041) which is available upon request from the Agricultural Research Service Collection NRRL, Peoria, IL: (see appendix for address)

METHODS:

1. Growth of bacteria in MMO broth supplemented with linalool.

   The broth MMO (100 ml) is placed in the sterile Nephelo culture flask. Three flasks are necessary.

   A. 100 ml of MMO broth supplemented with 0.2 ml of non-sterile linalool.

   B. 100 ml of MMO broth supplemented with 0.2 ml of non-sterile linalool and a faint bacterial inoculum (1 ml).

   C. 100 ml of MMO broth and just the bacterial inoculum alone (1 ml).

   The bacterial inoculum PFL 7.0 should be made by suspending a few loopfuls of bacteria from the surface of a Nutrient Agar (Difco) plate, into sterile distilled water (2 ml).

   Take initial absorbance readings on all flasks at 425 nm using the Spec. 20. Remember to cover the flask with aluminum foil before taking the absorbance reading at 425 nm.

Place each flask on the shaker ~300 rpm and let the flasks shake at 25°C.

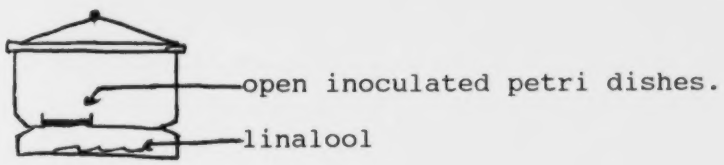
Plot absorbance 425 nm vs. time (h) for each flask.

Follow the growth of the bacteria and the disappearance of the linalool (oil) on the surface.

2. Growth of the bacteria on the agar surface on the linalool vapors.

After preparing the sterile MMO agar in the sterile petri dishes, streak some bacteria onto the surface of an MMO plate. This should be done using a bunsen burner and a sterile inoculating loop.

Pour some linalool into the bottom of the desiccating jar (10 ml). Place the open inoculated petri dish (lids off) into the jar and put the lid back on the desiccating jar.



Store the jar at 25°C for 24-72 hrs. Check the plates for the appearance of bacterial growth on the surface of the agar.

SAFETY CONSIDERATIONS:

1. Always wear disposable gloves when handling the MMO agar plates that are placed in the linalool jar.

2. Always wear disposable gloves when handling the linalool.

3. Open, close and store the desiccator - linalool in a fume hood.

CONCLUSIONS:

This experiment demonstrates the growth of a bacterial isolate on a branched hydrocarbon as a sole source of carbon and energy. This provides useful information when using specific bacterial strains to degrade toxic-recalcitrant hydrocarbons in nature.

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FUNGAL DEGRADATION OF POLYVINYL ACETATE

KEY WORDS: Plastic, Biodegradation, Xenobiotics.

BACKGROUND:

Plastics are synthetic compounds able to prevail in the environment because of their slow degradation or no degradation at all. This fact constitutes a potential hazard for the environment since these polymers tend to increase in great proportions. Microorganisms, mainly bacteria and fungi, have been described to degrade these xenobiotics, even in small proportions (Osmon *et al.*, 1970; Fields *et al.*, 1980; Garcia, 1988).

STATEMENT OF PROBLEM:

The purpose of this experiment is to obtain qualitative and quantitative evidence of the microbial degradation of a plastic in this case polyvinyl acetate (PVA) without any previous physical or chemical treatment. Microbial growth on the plastic as the only carbon source, increments of esterase levels and viscosity assays will be used as evidence of the degradation and utilization.

METHODS AND MATERIALS:

The following culture medium might be used:

Chemical	gm/L
Carbon source (PVA)	4.5
$(NH_4)_2\ SO_4$	1.0
KH_2PO_4	0.4
K_2HPO_4	3.2
$MgSO_4$	0.2
NaCl	0.1
$FeSO_4$	0.01

pH must be 5.0. However, any other mineral media supporting microbial growth may be used. Also notice that pH influences the type of microorganism selected.

Isolation of Experimental organism.

Samples of mud, soil or water from sites close to plastic pollution areas may be used as inoculants in the mineral medium (Note: Permission will need to be obtained to collect the soil. Caution should be used when collecting the soil and gloves should be worn). All incubations must be carried out at room temperature (22-25°C) with gyrotary agitation (150 rpm) until some growth is evident. Dilutions from this media are to be streaked into solid media (Agar free of inhibitors, 2% should be used) supplemented with PVA as the only carbon source. Selected strains were grown on slants and kept until use. If necessary, fungal spores, yeasts and bacteria may be counted and adjusted by means of a Newbaurer chamber. Mycelia may be determined by the increment in dry weight.(Reed, 1982)

Preparation of resting cells and crude enzyme solution.

The selected microorganisms may be harvested by centrifugation (10,000 rpm for 8 min), washed twice with 0.05 M phosphate buffer pH 6.0 and suspended in the same buffer, to be used as the resting cell suspensions. The extracts may be prepared either by maceration of mycelia in an ice cold bath or by sonication. In both cases, extracts are spun down at 5000 rpm for 20 min. The supernatant is used for the enzymatic activity assays.

Estimation of polyvinyl acetate degradation.

a) Qualitative: Strains showing consistent growth on the liquid media supplemented with PVA are to be streaked in the same solid media. Incubation must be at room temperature until mycelia start sporulating (approximately 3-4 days). At this point, sterile filter papers previously soaked in a ß naphtyl acetate solution were fixed at the sites of sporulation (Kersters and De Ley, 1971). The change in color from yellow to blue is interpreted as esterase activity during the breakdown of the polymer. Some blank plates (with other strains or no strain at all) should be run in the same way.

b) Quantitative: This method is based on the reactivity of ester groups with an alkaline hydroxylamine, which leads to a hydroxamic acid that forms a complex with ferric salts in an acid medium (Mann and Saunders, 1960). The color is proportional to the presence of ester groups, and can be followed spectrophotometrically at 530 nm (Rapport and Alonzo, 1955).

Measurement of viscosity. The viscosities of culture filtrates are to be measured with Ostwald viscosimeters calibrated at 25°C and the decrease in viscosity is interpreted as enzymatic activity.

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Note: At the end of the experiments, the student should provide: graphs (1 per strain) of the growth of microorganisms on PVA media. Photos or drawings of the colored reaction graph describing the esterase activity (As_{550}) vs concentration of ethyl acetate. Graph describing the loss of viscosity.

SIMULTANEOUS SACCHARIFICATION AND FERMENTATION OF BIOMASS TO ETHANOL

KEY WORDS: Fermentation, Ethanol, Biomass, Yeast Biomass.

BACKGROUND:

The simultaneous saccharification and fermentation (SSF) process for conversion of cellulose into ethanol was first studied by Takagi over ten years ago, and the process still shows great potential for economic production of ethanol. The SSF process employs a fermentative microorganism in combination with cellulase enzyme to minimize accumulation of sugars in the fermenter. As a result, inhibition of the enzyme by its product sugars is reduced, and higher hydrolysis rates and yields are possible than for straight saccharification. However, to produce ethanol from the SSF process that is competitive in price with the cost of petroleum-derived fuels, hydrolysis yields must be increased, enzyme costs must be reduced, and ethanol production rates must be improved.

Cellobiose fermenting yeast have also been studied because additional ß-glucosidase activity can speed up the SSF reaction. The importance of end-product inhibition of the cellulase enzymes during cellulose hydrolysis has been modeled. Some publications discuss the advantage of the cellobiose fermenting yeasts in decreasing end-product inhibition of cellobiose to the cellulase enzyme. In general, *S. cerevisiae*, a strong thermotolerant glucose fermenter with a fast rate of fermentation, has been found to perform well if the enzyme preparation is high ß-glucosidase.

An important element in the SSF process is the choice of substrate. Several cellulosic substrates have been evaluated in the SSF process including sugar cane bagasse, rice straw, wheat straw, wood fractions and paper mill by-products. A pretreatment step is necessary before these substrates can be used in SSF. For the purposes of learning the SSF process, Whatman #1 paper, shredded and blended to a pulp, or sigmacell-50 cellulose are good substrates to use.

STATEMENT OF PROBLEM:

The problem is to determine which substrate will result in the highest yields of ethanol.

MATERIALS:

S. cerevisiae stain can be cultured from Red Star Brewers Yeast, or from the Northern Regional Research Laboratory (NRRL), USDA, Peoria, IL. Cellulose and Chemicals can be purchased from the Sigma Chemical Company, St. Louis, MO, and yeast extract and peptone growth media from Difco, Detroit, MI. Cellulase enzyme came from Genencor Inc., San Francisco, CA, and ß-glucosidase (Novozyme - 188) from NOVO Laboratories, Inc., Wilton, CT. Shaker flask, 250 ml Pyrex graduated vessels, and Braun Biostat V6 fermentation vessels were used for the fermentations.

SSF Fermentation

Media:

10 or 15% Cellulose
1% Yeast Extract
2% Peptone
1% Glucose
0.5% Cellobiose
5 ml/L Lipids*
2 ml/L Antibiotics*

*Lipids - Stock:	50 gm Tween 80 50 mg Ergosterol

1. Dissolve ergosterol in minimal volume of 95% ethanol (2-3 ml).

2. Mix ergosterol/ethanol solution into 50 gm Tween 80 (o.6% unesterified oleic acid).

3. Evacuate and flush with N_2. Final medium concentration = Ergosterol 5 ml/L, Oleic Acid 30 ml/L.

*Antibiotics	Penicillin 10 mg/L (16,500 U) Streptomycin 10 mg/L
Stock: (Filter Sterilize)	500 mg/ Pen 500 mg/ Strep 100 ml H_2O

Large Scale SSF: Add media to 6 L vessel with 1 L H_2O already in it. Bring volume up to 2,500 ml to leave room for inocula. Mix media in fermentor and add lipids. Clamp off hoses etc. and autoclave for 35-40 min. Let cool, add antibiotics, check pH, add enzyme, add inocula and bring volume up to 3 L mark with sterile H_2O.

Small Scale SSF: Use 250 ml erlenmyer flask with fermentation set up of cork and CO_2 trap. Add cellulose and autoclave for 15-20 min. Make a 2x stock solution of YP media with lipids (autoclave) and add antibiotic. Use 50 ml of this 2x stock, and calculate the amount of enzyme and H_2O, according to the displacement of cellulose and inoculum volume. The cellulose displacement of 10% in 100 mls is 6 ml, for 15% it is 9 ml. Add everything under the hood, put water in the water trap tube and start shaking.

Sampling: In large scale, it is important to release a little of the media through the sample port before sampling to obtain a uniform sample. In small scale, it is important to swirl the flask, suspending all of the cellulose that may have settled before pipeting out the sample.

METHODS:

Shaker flask SSFs were carried out in 250 ml flasks outfitted with stoppers constructed to vent CO_2 through a water trap. These flasks contained 100 ml of fermentation broth and were agitated at 150 rpm in a shaker incubator at 37°C. A 1% yeast extract and 2% peptone (w/v) media was used with a substrate loading of 7.5% (w/v) cellulose. A lipid mixture of ergosterol (5mg/L) and oleic acid (30mg/L) was added to the media for improved ethanol yield. Also, penicillin and streptomycin at 10 mg/L were used to minimize bacterial contamination. The inocula were grown in a shaker flask with (YP) media and 2% (w/v) glucose at 37°C, and a 1/10 (v/v) yeast culture to total volume of media was added to the fermentation. The substrate was autoclaved in fermentation flasks, and sterile media, lipids, antibiotics and enzyme were added before the inoculum.

Ethanol concentrations in the supernate were measured by gas chromatography using a Porapak Q80/100 column. The internal standard was 4% isopropanol. For the larger scale 3 L SSFs, residual sugars (glucose and cellobiose) were determined as glucose by incubation of the sample with 2 mg/mL almond extract ß-glucosidase from Sigma for 1 hour at 37°C, and total sugars were measured on the model 27 glucose analyzer from Yellow Springs Instruments, Yellow Springs, OH. Viable cell densities were measured as colony forming units (CFU) by plating serial dilutions on YPD or YPC plates.

Alternatively, kits may be purchased for use with a spectrophotometer in order to determine ethanol concentrations.

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SOLUBILIZATION OF FISH PROTEINS BY IMMOBILIZED MICROBIAL CELLS

KEY WORDS: Proteases, Protein solubilization.

BACKGROUND:

Proteases have several applications in the food industry which include meat tenderization, cheese ripening, chill-proofing of beer and bread making. In the seafood industry, the enzymes are used for modification of functionality of fish protein concentrate, preparation of fish protein hydrolysate and recovery of protein from miscellaneous, low-priced fishes, which constitute about 30% of total world fish production. Microorganisms that secrete significant quantities of extracellular proteases may be inexpensive sources of proteases for the purpose.

STATEMENT OF PROBLEM:

To produce a fish meat powder with enhanced solubility by using immobilized microbial cells. Different species of protease producing bacteria could be compared for efficiences of solubilization.

MATERIALS:

Fish: Any cheap variety of fish
Microorganisms: *Bacillus megaterium* or *Bacillus licheniformis*
Chemicals and reagents: Sodium alginate, Calcium chloride, Tris buffer, 0.05 M, pH 7.5; Nutrient broth (Difco) Nessler reagent, Folin reagent (optional), Sodium hydroxide, 2N; sulfuric acid, 5N.
Protein assay kits may be obtained from companies such as Sigma Chemical Co.
Equipment: Centrifuge, pH wand, Water bath, Steam cooker, deboning machine (optional).

METHODS:

The fish is cut open, eviscerated and washed in tap water. The meat may be collected from the dressed fish by passing through a deboning machine. A partially deodorized meat is prepared by cooking the fish or deboned meat (1 kg lot) for 15 min in 3 L of water containing 1% acetic acid. The cooked meat is separated and pressed to obtain a partially deodorized preparation. If no deboning machine is used, bones should be removed from the cooked meat by hand. The meat is stored at -10°C until used (Venugopal *et al*, 1989.).

Immobilized microbial cells are prepared as described by Venugopal *et al* (1989). The bacterium is grown in nutrient broth. The cells are harvested by centrifugation and washed once with 0.05 M Tris HCl buffer, pH 7.5. The cell pellet (15 g) is mixed with 75 ml of 1.5% (w/v) sodium alginate. The mixture is pumped through a hypodermic needle into cold 0.1 M $CaCl_2$. The beads are cured for 3 h, washed and stored at 4°C in Tris buffer, pH 7.5 until used.

The beads (4 g) are suspended in 50 ml of 10% fish homogenate in water. The mixture is incubated at 50°C in a water bath with gentle shaking. Using a pH wand (Cole Palmer, Chicago), the pH of the slurry is maintained at 8.0 throughout the experiment by drop wise addition of 2 N NaOH. At intervals, aliquots of the slurry are withdrawn, centrifuged at 5900xg for 15 min and the supernatant is used for nitrogen estimation, which is taken as an index of protein solubilization.

Aliquots of the supernatants are digested on a sand bath with 5 N H_2SO_4 containing traces of sodium selenite. When the solution has become clear, it is cooled, diluted with distilled water and known volume is used for an estimation of nitrogen by Nesslierzation using

ammonium chloride as the standard (Umbreit, *et al*, 1959.). Alternatively, the content of tyrosine in the supernatant could also be measured using Folin's reagent (Lowry, *et al*, 1951.). Fig. 1 gives a typical solubilization rate of fish meat by the process.

The treated slurry could be dried to obtain a fish powder with enhanced solubility.

COMMENTS:

In order to recover the beads from the slurry after the treatment, the immobilized cells could be held in a beads holding assembly made of perforated perspex cylinder containing inside discs for distribution of the beads (Fig. 2). The beads holder is attached to a motor for rotation at a slow speed (30 rpm) in the slurry. A perspex piece attached to the bottom of the beads holder helps to stir the sediments of the meat.

Apart from the organisms mentioned above, other protease producing non-pathogenic organisms could also be used. Use of organisms genetically engineered to produce enhanced levels of protease (2) can increase the extent of solubilization. Further, the process may be examined for solubilization of proteins from other sources like slaughter house and poultry processing wastes.

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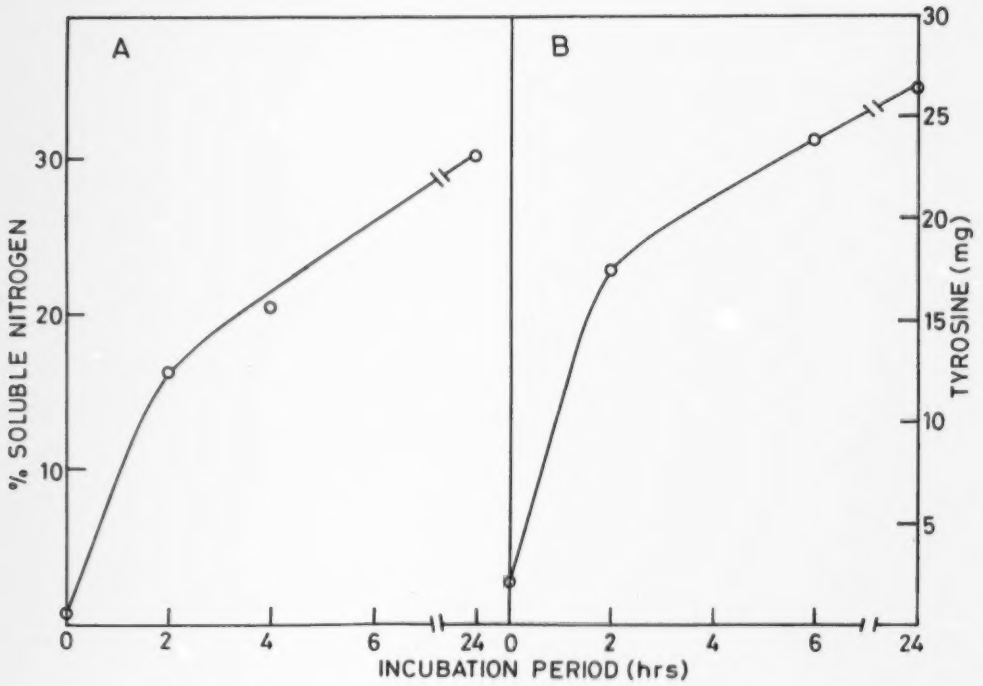

Figure 1. Rate of solubilization of fish meat by immobilized *B. megaterium*.

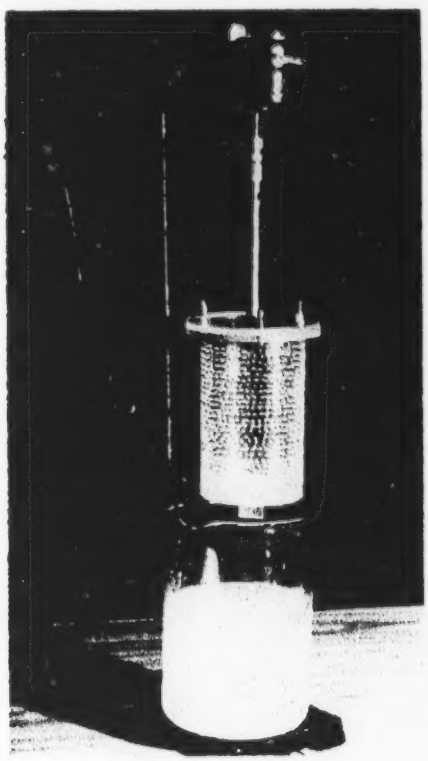

Fig. 2 Beads holding assembly.

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PREPARATION OF CHITIN AND CHITOSANS FROM CRUSTACEAN WASTE BY ENZYMATIC METHODS

KEY WORDS: Chitin, Chitosans.

BACKGROUND:

Chitin, the second most abundant compound on earth, is found mainly in marine invertebrates, insects, fungi and yeasts and constitutes a waste disposal problem for the food and biotechnology industries (Rehvah-Moissey and Carroad, 1981). Chitosan is a positively charged polyelectrolyte derived from chitin by deacetylation using chemical or enzymatic/microbiological methods. The polyelectrolyte nature of chitosan makes it a more valuable product than chitin for applications as diverse as (a) flocculating agent for the clarification of fruit juices or treatment of fish processing waste water, (b) dehydrating agent for concentration of alcoholic beverages and industrial alcohol, (c) hemostatic blood coagulant, (d) immobilization of enzymes and whole cells, (e) biomass recovery, and (f) functional ingredient for other food and pharmaceutical applications (Knorr and Sinskey, 1985).

Previous studies with chitin and chitosans indicate that chitosans from different sources have different properties. The variations may be due to the source material, isolation procedures employed for chitin production and subsequent processing to chitosan. A knowledge of the properties of chitosans is important for various reasons: (i) it is useful in defining the quality of the product obtained from chitosans (eg. flocculants, biodegradable membranes, chromatographic aids, textile and paper additives, etc.); (ii) it will facilitate the formulation of products with more consistent reproducible and well defined properties; and (iii) it will facilitate development of new chitosans with improved performances or from novel sources.

STATEMENT OF PROBLEM:

The overall project objectives are to (i) convert crustacean waste to chitin, and (ii) establish that enzymatic methods produce better chitosans with consistent and well defined properties as compared with chemical methods.

METHODS AND MATERIALS:

Crustacean waste for use as source material for recovery of pigment and protein (carotenoprotein), chitin and chitosans or flavor extracts may be obtained from fish processing plants and/or seafood restaurants. The residue after recovery of carotenoprotein from waste (Simpson and Haard, 1985) may also serve as source material for recovery of chitin. Other materials (chemicals) include hydrochloric acid, monobasic and dibasic sodium phosphate, pentane, sodium hydroxide, sodium hypochlorite, trisodium ethylenediaminetetraacetate.

Preparation of chitin and chitosans:

The residue after recovery of carotenoprotein is used to prepare chitin by a modified form of the procedure by No *et al.*, (1989), outlined in Fig. 1. Other modifications to the scheme outlined in Fig. 1 that may be employed include:

(a) deproteinization with 3.5% NaOH for 2 h at 60°C, or

(b) demineralization with 0.5M Na_3EDTA, pH 7.7, at ~23°C for 3 h.

Chitin content in residue, carotenoprotein and product recovered by the flow process summarized in Fig. 1, is determined by the method of Spinelli *et al.*, (1974). Acetyl value and

solubility of chitin is determined by the method No *et al.*, (1989) and compared with those of commercially available chitin.

Chitin prepared by the process in Fig. 1, is converted to chitosans with a culture of *Mucor rouxii* (DSM 1191) according to the procedure described by Knorr and Klein (1986). The microorganism is cultured in a medium made up of 30 g malt extract, 15 g agar and 3 g peptone per l, subcultured into 50 mL of liquid medium (with or without 1 g of dried chitin) and incubated at ~25°C on a reciprocal shaker. The degree of acetylation and viscosity of chitosans produced in mycelia of *M. rouxii* are determined using the spectrophotometric and viscometric procedures of Muzzarelli and Rochetti (1985), and compared with those of commercially available chitosans.

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Figure 1

Scheme for Recovery of Chitin from Crustacean Waste

CRUSTACEAN WASTE

(Residue after Recovery of Carotenoprotein)

Drying (60°C, in vacuo)

Grinding, Seiving & Washing

DEPROTEINIZATION

(2% NaOH for 6 h; Temp. ~23°C; solids to solvent = 1 : 15 w/v)

Filtration

Deproteinized Crustacean Waste

Washing

DEMINERALIZATION

(1M HCl for 30 min at ~23°C; solids to solvent = 1 : 15 w/v)

Filtration & Washing

EXTRACTION OF PIGMENTS WITH ACETONE

Washing & Drying (60°C, in vacuo)

BLEACHING

(0.3% NaOCl for 6 h at ~23°C; solids : solvent = 1 : 15 w/v)

Washing & Drying (60°C, in vacuo)

CHITIN

ENHANCING VALUE OF CRUSTACEAN WASTE BY ENZYMATIC METHODS

KEY WORDS: Chitin, Chitosans, *Mucor*, Biodegradation.

BACKGROUND:

In terms of dollars, the crustacean fishery (including shrimp, lobster and crab) is one of the most valuable food industries in North America, and accounts for more than one billion dollars of the total fish landed each year (Anon, 1989). In processing these crustacean species, the head, viscera and carapace are removed by semi-mechanized peeling operations and the waste generated is about 70% of the landed catch, which is conservatively estimated at 500,000 metric tons. However, with more stringent environmental regulations and standards regarding the disposal of crustacean waste, there is a renewal of interest by the industry for the conversion of these waste materials into useful by-products. Crustacean offal is a rich source of (i) protein and energy for use as feed for fish reared in captivity; and (ii) carotenoid pigments that may find use as colorant for imparting orange-red color to flesh of pen-reared salmonids or as food colorant for surimi-based seafood analogs and/or soups. In this regard processing crustacean waste into useful by-products will not only result in profits for the processor, but will alleviate waste disposal problems and associated environmental concerns.

STATEMENT OF PROBLEM:

This project aims at (i) recovering a product (carotenoprotein) enriched in protein and fat, but depleted of ash and chitin, and (ii) confirming that the recovered product has great potential for use as feed supplement for cultured salmonids.

METHODS AND MATERIALS:

Crustacean waste for use as source material for recovery of pigment and protein (carotenoprotein) may be obtained from fish processing plants and/or seafood restaurants. Other materials (chemicals) include acetone, ammonium sulfare, bovine trypsin (type III, Sigma Chem. Co.)), hydrochloric acid, monobasic and dibasic sodium phosphate, petroleum ether, pentane and trisodium ethylenediaminetetraacetate.

Recovery of carotenoprotein:

Carotenoprotein from crustacean waste is recovered by the method of Simpson and Haard (1985), summarized in the flow process diagram (Fig. 1). Various modifications of the extraction scheme that may be employed include the following:

(a) Exclusion of EDTA from process; This is achieved by homogenization of the waste material at moderate speed in 0.05 M phosphate buffer (pH 7.7) followed by digestion with trypsin at (i) 37°C for 1 hr, or (ii) 50°C for 30 min.
(b) Exclusion of $(NH_4)_2SO_4$ from the process; This is achieved through isoelectric precipitation, by adjusting pH to 4.6 with 1 M HCl and allowing fraction to settle at ambient temperature for 6 hrs.

The material recovered after the centrifugation step may be dialyzed to remove salts and dehydrated into a stable dry powder by freeze drying, tray drying, foam mat drying or spray drying.

Carotenoprotein thus recovered, is analyzed for moisture, total nitrogen, crude fat and ash by standard AOAC (1984) procedures. Crude protein is calculated from total nitrogen from N x 6.25. Total astaxanthin is determined by the method of Saito and Regier (1971) as modified for use with Pandulus borealis, Kroyer (Simpson and Haard, 1985). The absorbance

of the extract, appropriately diluted, is measured at 468 nm and used to calculate the concentration of the carotenoid pigments present in the sample (Simpson and Haard, 1985). Feeding trials with salmonids is carried out as follows: two groups of salmon or rainbow trout are given rations containing carotenoprotein at different levels of incorporation. A third group of fish is given rations supplemented with an artificial colorant, and a fourth group is fed rations devoid of colorants. The caloric and nutrient content of rations are standardized for the four test diets. Fish are sacrificed after six weeks on test diet and on the weight gain, mortality and any evidence of mortality recorded and statistically analyzed. The color of raw and cooked fish muscle is determined by colorimetry, sensory panels and by determination of total astaxanthin content.

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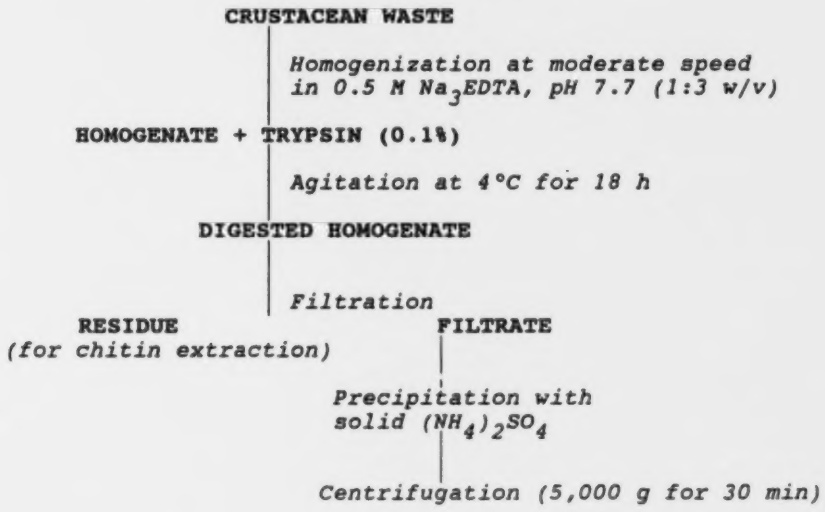
Figure 1

Scheme for Recovery of Carotenoprotein



VEGETABLE OIL EXTRACTION BY ENZYMATIC REACTIONS

KEY WORDS: Oil extraction, Enzymes.

BACKGROUND:

Currently the main processes for oil extraction from seeds or other vegetables are the hydraulic process, using a wide variety of equipment, mainly expellers, and the solvent extraction process, in which hexane or other solvents are used to dissolve and extract oil from the raw materials.

During this decade, the advances in enzymology have resulted in the availability of enzymes active towards many cellular components such as cellulases, hemicellulases, pectinases, xilanases, ß-glucanases, proteases, amylases, etc.

Two processes have been developed in our laboratory: the enzymatic extraction of coconut oil (2) and the enzymatic extraction of avocado oil (1). In both cases, the results have been interesting enough to pursue agroindustrial applications. A cellulase developed by Gennecor has also been proposed to increase the olive oil extraction yield.

The oil is usually inside of vegetative cells, linked with other macromolecules, so that upon partial hydrolysis the extraction is enhanced. Since these macromolecules may include proteins and a wide variety of polysaccharides, the hydrolysis is only possible if the appropriate enzymes are selected. The structure of vegetative cells is not uniform. It changes with maturity and is different between species. Each case is different and has to be studied separately.

STATEMENT OF PROBLEM:

You are to develop a process for oil extraction of a given raw material (at least 20% of oil), selected with a proper justification (regional availability, economical impact, waste disposal problems, etc.). The process has to be defined mainly in terms of:

a) Type and concentration of the required enzymes

b) Reaction conditions (dilution, pH and temperature)

c) Oil recovery steps (filtration, centrifugation,..)

A statistical design of experiments should be the base to optimize the process. When possible, oil extraction yield may be compared with the one obtained in the actual extraction process.

METHODS AND MATERIALS:

Raw materials such as soybean and sunflower, should be available in the region. It can be characterized by proximate analysis, measuring protein, ash, carbohydrate, fiber and oil content.

Mixers or grinders could be used if a pretreatment of the raw material is needed.

The enzymatic reaction should be carried out in reaction vessels provided with agitation and temperature control.

After the enzymatic reaction, you may need a press or a centrifuge for oil recovery.

Finally, the extracted oil can be quantified by weight or by volume.

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CELLULAR PHYSIOLOGY

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MEASUREMENT OF THE EFFECT OF THE PRESENCE OF RECOMBINANT DNA ON THE GROWTH OF BACTERIA

KEY WORDS: Plasmid, Growth curve, Klett meter, Spectrophotometer.

BACKGROUND:

The oldest (and still one of the most widely used) applications of "recombinant DNA technology" involves the "genetic engineering" of bacteria to produce useful products more easily and cheaply than would otherwise be possible. These products include enzymes, vaccines, and growth factors.

Genetic engineering of bacteria involves isolating a gene, that is, a piece of DNA (called the "insert"), which encodes a product of interest, and recombining it with a second piece of DNA (a "vector") which can reproduce in the bacterium in question. This vector-insert combination (the "recombinant DNA molecule" or "recombinant plasmid") is then introduced into the "host" bacterial cells where it replicates, the insert sequence directing synthesis of the desired product. The host bacterium-recombinant DNA system is called a "recombinant bacterium" or a "plasmid-bearing cell."

It is evident from the preceding discussion that a recombinant bacterium can make the product of interest only as long as it maintains the recombinant plasmid. Loss of recombinant DNA from cells of a culture of recombinant bacteria can, in fact, be a serious problem. It has two main causes. First is that there is a finite possibility when a recombinant cell divides that one of the daughter cells will not have a recombinant plasmid. Secondly, cells without recombinant plasmids grow better, in general, than plasmid-bearing cells. Thus, once even a single plasmid-free cell is formed, it can outgrow the remaining plasmid-bearing cells; this results, eventually, in a culture that is comprised completely of plasmid-free cells.

STATEMENT OF PROBLEM:

This experiment is designed to demonstrate the second phenomenon discussed in the preceding paragraph, namely the growth disadvantage that plasmid-bearing cells have compared with plasmid-free cells. In addition, it will show that, in general, this disadvantage increases as the size of the recombinant plasmid increases.

METHODS AND MATERIALS:

The growth of three strains of the bacterium *E. coli* (two recombinant [called Xf3 and BS5] and one non-recombinant [called JM103]) is to be measured; these strains can be obtained from the submitting author. Supplies needed include tryptone, yeast extract, sodium chloride, sodium hydroxide, and ampicillin (all of which might be obtained from a local clinical lab, college or university biology department) to prepare the growth medium for the cells; and 1 ml and 5 ml pipets, a bacteriological "loop", and 25 ml and 250 ml Erlenmeyer flasks with sterilizable caps (probably available from a high school chemistry lab) for transferring and growing cells. Equipment you will need includes a pH meter (for medium preparation), an autoclave (for sterilizing medium, pipets, and flasks), a bunsen burner (for sterile transfer of cells), an air or water shaker (for growing cells), and a Klett meter with Klett tubes or spectrophotometer and cuvettes (for monitoring cell growth). Some of the equipment may be available at your school, but some may have to be borrowed from a clinical or university lab. Methods of sterile transfer of cells and theory and operation of the Klett meter are best learned by visiting a local clinical, college, or university lab.

The experiment will involve growing up small volumes ("inocula") of each of the three strains overnight in LB media. Xf3 and BS5 should be grown in LB containing 35-50 μg/ml of ampicillin in or to maintain their plasmids, followed by dilution of a small amount of each

inoculum into fresh medium the next day. As the cells grow, the medium becomes more turbid; this growth is quantitated by removing samples periodically from each flask and measuring their turbidity with the Klett meter. A plot of $\log_{10}$ of turbidity versus time after inoculation (like Figure 1, which plots the $\log_{10}$ of the equivalent of turbidity, namely cell number, versus time) is the most common way to present such data. The experiment should take several hours of preparation time and then about one hour the first day, and about 8-9 hours the second day of the actual experiment.

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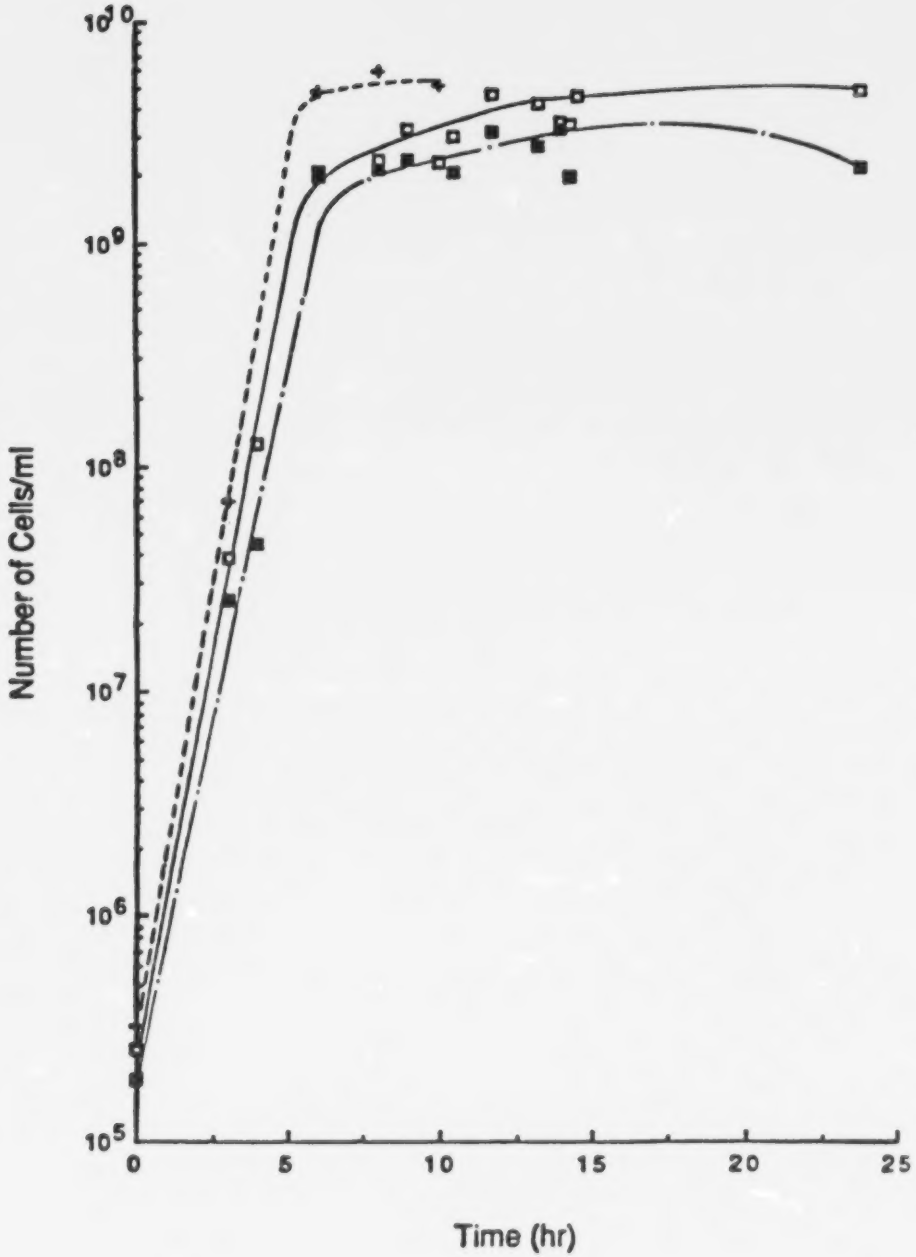

Growth of E. coli strains JM103 (plasmid-free), Xf3 (contains a 2.7 kb plasmid), and BS5 (contains an 8.7 kb plasmid) in LB medium. (+---+) JM103; (□——□) Xf3; (■–·–◀) BS5. Figure courtesy of Mahvash Khosravi.

THE USE OF A PROTOZOAN TO HELP DETERMINE IF CERTAIN CHEMICALS MAY BE IRRITATING TO AN ANIMAL'S EYE

KEY WORDS: Draize test, *Tetrahymena*, Product safety test, Effective dose.

BACKGROUND:

In the early part of this century, many people received injuries to their eyes from household chemicals such as soaps and detergents as well as from chemicals used in cosmetics and industry. Because of a public outcry for product safety testing, the federal government, over a period of time, formulated various laws and regulations that were designed to help assure that certain consumer products would not cause these injuries.

In 1944, Dr. John Draize and his colleagues designed a test to assess the ability of chemicals (or commercial products) to cause irritation to the eye. In the so-called Draize test, a small amount of the test product is placed in the area between the eyelid and the cornea (the clear part of the eye), and the eye is observed for 3 to 7 days. Using a numerical scale, the degree of damage that the product has caused to different parts of the eye is recorded. If there is significant damage to the rabbit's eye, the product is presumed to be able to cause significant damage to the human eye.

There has been a good deal of criticism of the Draize test, some of which has to do with the ethics of using rabbits to serve as a model for human eye irritation studies.

STATEMENT OF PROBLEM:

To help decrease the use of rabbits in product safety testing, we wish to develop a test that does not require a vertebrate animal, is painless, easy to perform, rapid, inexpensive, and can give good correlation with the Draize test.

METHODS AND MATERIALS:

Consider using a sterile culture of a protozoan such at *Tetrahymena*, since its biological characteristics are very well known and it can swim using cilia. This is important, since if the cell does not swim in the presence of a chemical, then the chemical is toxic to the cell. You might also consider other single-celled organisms such as *Paramecium*. These cells have no nervous system to speak of and cannot experience pain. They are readily available from sources such as Carolina Biological Supply Company or the American Type Culture Collection.

Other materials needed are a microscope, a microscope slide (preferably with a well in the center), glass microscope cover slips, different sized test tubes, a Bunsen burner, 0.4% sodium chloride, non-sterile pipets for making chemical dilutions, a bacteriological inoculating loop, culture medium, the ability to sterilize the culture medium (an autoclave is best, although you might try boiling it as if you were canning fruits) and sterile 2 ml pipets. You will also need test chemicals such as commercial shampoos, detergents, and household cleaners.

Begin by placing 10-15 ml of culture medium in test tubes and sterilizing them. After cooling at room temperature, and using sterile technique, inoculate approximately 0.5 ml of *Tetrahymena* suspension from the stock culture into each of three test tubes. Let them sit in the dark at room temperature for approximately 72 hours. This allows the culture to grow and gives you sufficient organisms for testing.

Using only 1 of the 3 cultures you grew, place one drop of *Tetrahymena* suspension (the actual volume should be measured with a pipet) in a small test tube. This does not have

to be done in a sterile manner. Then place the same volume of 0.4% sodium chloride in the same test tube and mix the two together for a few seconds. With the inoculating loop, place a loopful of the mixture on the microscope coverglass, carefully invert it over the slide with the well in it, and place it under the microscope. View the slide under 40 to 100 magnification. The 0.4% sodium chloride should not harm the cells, and you should see them rapidly swimming in the drop of water. Carefully note how they almost never stop and how they appear to swim in random patterns. You might want to just take a loopful of *Tetrahymena* out of the culture tube to see how they move without the sodium chloride. If the sodium chloride is not allowing the cells to swim normally, something is wrong and the test should not proceed. The sodium chloride is called the "negative control." Put the inoculating loop through the Bunsen burner after you have transferred the cells onto the microscope slide.

Now take the chemical you wish to test. Your goal is to find the concentration of that chemical that stops only 10% of the cells from moving normally, as compared to the negative control. This is called the ED_{10} (effective dose, 10%). Remember, the negative control should have about 100% normal movement. Do this by making dilutions of the chemical (e.g. 1:10, 1:50, 1:100) with the 0.4% saline and then mixing one drop of the diluted chemical with one drop of the *Tetrahymena* and observing the motion of the cells under the microscope as you did with the negative control. Begin by making a dilution such as 1:50, and then make big jumps such as 1:200 if the cells are not moving or 1:10 if they are all moving. You are trying to approximate where the ED_{10} should be, then you can make dilutions that you think are closer to the actual ED_{10}. With some detergents, dilutions of 1:2000 or greater are not unusual. You do not have to be exact.

Do not worry about how fast the cells are moving, and do not concern yourself with cells that may be temporarily stuck at the water-air interface. The abnormal movement you are looking for are cells that do not move at all, or cells that move in tight little circles and never go anywhere. Make your decision as to the percentage of normally moving cells after 2 min of observation.

The second culture that you have grown can be used to try to duplicate your results, and the third culture can be used to inoculate other test tubes for future use.

After you have learned the technique you can use any chemical that you have already tested as a "positive control." That is, you should test that chemical at least once during each test day to make sure that the cells are responding the same way each time. It is an excellent safeguard when your test series may extend over many days, weeks or even months.

You can now compare the toxicity of similar products to the protozoan culture by "rank ordering" them. The greater the dilution you have to make to reach the ED_{10}, the more toxic the chemical is to the cells. The more toxic it is to the cells, the more irritating it should be to the rabbit eye. Compare alcohols to alcohols, detergents to detergents, etc. Your results may not be valid if you try to compare different classes of products to each other.

How can you determine if your results would compare to the actual testing of the chemicals in rabbit eyes? The only truly accurate way would be to test the exact same chemicals by the Draize test and statistically compare the results. Another way of approaching the problem is to do library research that compares past experience with the Draize and related tests with the chemicals you have tested. This is not very difficult with pure products such as ethyl alcohol, acetone and the like, but it can be frustrating when you try to find information on commercially formulated products. It is somewhat comforting to know that controlled testing has indicated that *Tetrahymena* is a reasonable indicator organism for determining if many commercial products would be toxic to the eye of the rabbit.

Tetrahymena Culture Media

I. A. Liver Powder (Difco Co.) #0133 — 2.0 g
B. Saccharomyces Cerevisiae (baker's yeast type II) (Sigma Chemical Co. YSC-2) — 2.0 g
C. Soy Lechithin (Sigma) — 0.02 g
D. Mix together in large flask with 2 L of distilled water. Stir while heating to ~90°C. Place into smaller bottles and sterilize. Store in refrigerator. Transfer cells to fresh medium every 2 weeks.

OR

II. A. Puree a piece of fresh beef liver with a little distilled water.
B. Weigh out 7.4 g of the puree.
C. Add yeast and soy lechithin as above.
D. Add 2 L of distilled water. Adjust pH to 7.5-7.8 with hydrochloric acid. Boil about 15 minutes, place into smaller bottles, sterilize and store in refrigerator. Transfer cells to fresh medium every 2 weeks.

OR

III. A. Proteose peptone (Difco #0120) — 5.0 g
B. Tryptone — 5.0 g
C. K_2HPO_4 — 0.2 g
D. Distilled water — 1.0 L
E. Adjust pH to 7.2. Stir well. Place in smaller tubes. Transfer to fresh medium every week. Cells may not grow quite as well as in other media.

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Interested students can contact Dr. Silverman directly at (614) 292-3382. He will be pleased to provide research publications and other information. As the field of *in vitro* toxicology is fairly new, student level publications are not available.

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HORMONAL INDUCTION OF CELLULAR REPLICATION AND METABOLISM

KEY WORDS: Insulin, Tissue Culture, Fibroblast, DNA Protein.

BACKGROUND:

One of the primary goals of the industry of animal biotechnology is to promote faster growth and development of farm animals for use by animal agriculture. One of the major ways in which new products which are developed in industry laboratories are evaluated is by their action on various "screens". Screens are tests which permit the rapid determination of the activity of an unknown compound. These tests must be simple, cost-effective and, most importantly, quick. Many companies must screen several hundred new compounds a week; therefore, the screens must take little time or a tremendous backlog will develop. Many of these tests are strictly of a chemical nature, while other screens require the determination of a biological activity like cell replication.

The ability to stimulate cell replication (a mitogenic response) is one of the most important tests currently being utilized for screening in the industry of animal biotechnology. A mitogenic response can be measured several ways. One can actually count the number of cells that are present in an experimental versus control group; unfortunately, this is labor-intensive and not quick. An easier method is to quantitate the amount of DNA that results from exposure to an experimental versus exposure to a control treatment. An increase in the total amount of DNA due to the experimental treatment would indicate an increase in the total number of cells, since the amount of DNA/cell is relatively constant.

The amount of protein that develops in a population of cells exposed to various treatments has also been utilized to determine cell replication. However, protein content has certain flaws when utilized for this measurement. An increase in cell protein could indicate an increase in cell number, but it could also indicate an increase in the size of each cell. Cells become larger as many tissues grow without any change in the number of cells. Therefore, protein content is usually a better indicator of cell size or metabolism. The protein data is usually corrected for the number of cells which contain the amount of protein. Protein to DNA ratios will tell you if one treatment results in the production of more protein than another treatment while correcting for any potential differences in cell number. The DNA content will tell you if there is a difference in cell number.

STATEMENT OF PROBLEM:

Insulin is one of the major hormones which influences the development of cells within the body. The present experiment was designed to compare the actions of insulin at two different concentrations upon the metabolism and replication of cells utilizing tests for protein and DNA as screening tools.

METHODS AND MATERIALS:

Incubator

a. Incubator or oven which can be maintained at 37°C
b. Tank of 95% air, 5% CO_2
c. Pan of water

Cell culture

a. 3T3 fibroblasts - acquire form the ATCC
b. Dulbecco's Modified Eagle's Medium (DMEM) - Sigma
c. Fetal bovine serum (FBS) - Sigma
d. Insulin - Sigma
e. Sterile pipets
f. Pipeting devices - Finnpipet, Pipetman, etc.
g. Disposable sterile filtration device - Fisher
h. Disposable tissue culture dishes (35mm)
i. Sterile bottles for media (can be disposable plasticware)

Assays

a. Spectrophotometer
b. Protein assay reagents
c. Test tubes
d. Pipeting devices
e. DNA assay reagents
f. Fluorometer
g. Microcentrifuge
h. Microcentrifuge tubes
i. Rubber policeman

All supplies and materials can be purchased from major scientific supply companies such as Fisher Scientific or Sigma Chemical Co. through your local school system. The cells for use in these experiments are a cell line which can be obtained by your local school system from the American Type Culture Collection (ATCC, 12301 Parklawn Dr., Rockville, MD 20852). No animals will be utilized for these experiments and obtaining these cells does not involve their collection from animals.

An incubator will be necessary for the growth of the cells in an artificial environment, in culture. A local hospital or college should have an incubator available in their Departments of Biology or Cell Biology. A pan of water must be placed in the bottom of the chamber to provide humidity to the atmosphere. A tank of 95% air, 5% CO_2 will be necessary to provide a constant flow of an appropriate atmosphere to the cells. The gas should be supplied through flexible tubing into the incubator by way of a hole in the wall of the incubator.

Once the cells have been delivered, they should be immediately diluted in the appropriate medium and seeded onto 35 mm tissue culture dishes according to the documentation provided by the ATCC. The medium is replaced the next day with DMEM containing 10% FBS. This day is designated day 1 of culture. The medium must be sterilized before usage. This is most easily done with a sterile, disposable filtration device and a sterile bottle. Follow the recommendations provided with the device to correctly sterilize the solution. Use sterile pipets to add the serum to the medium.

Safety Statement

Because mammalian cells and culture medium can harbor pathogens, it is important that all cell cultures be sterilized before disposal. Gloves should be worn when working with cultures. Good aseptic technique should be used at all times.

The medium is changed on day 3 of culture. The medium is replaced with test media when the cells completely cover the tissue culture dishes. The treatment media all contain DMEM with 1% FBS. The three treatments are: 1) 0 nM insulin, 2) 1 nM insulin, 3) 1000 nM insulin. The cells are exposed to the treatment media for 5-7 days with changes every 2-3 days.

The cells are harvested from the tissue culture dishes using a rubber policeman to scrape the cells from the dishes and into microcentrifuge tubes. Rinse the dishes with 1 ml of .9% saline to remove any residual cells. The tubes are rapidly frozen in a mixture of ethanol and dry ice. Be very careful, as this solution is extremely cold. Please wear goggles for this step. Repeat freezing and thawing the cells rapidly for 3 cycles. The tubes are centrifuged at 10,000 x g for 5 min. This microcentrifuge should be available at your local hospital or college. The liquid portion of the lysed cells is used for determination of protein content according to the method of Bradford (1976) and using a spectrophotometer. The pellet at the bottom of the tubes is used for determination of the DNA content according to the procedures of Kissane and Robins (1958) and utilizing a fluorometer. The specifics of these two procedures can be provided on written request.

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BIOTECHNOLOGY INFORMATION SOURCES

American Biotechnology Association
P.O. Box 8258
Princeton, N.J. 08543-8258
phone 609-896-8258

American Chemical Society
1155 16th St., NW
Washington, D.C. 20036
phone 202-872-4600

American Society for Microbiology
1325 Masssachusetts Ave., NW
Washington, D.C. 20005
phone 202-737-3600

Association of Biotechnology Companies
1120 Vermont Ave., NW
Suite 601
Washington, D.C. 20005
phone 202-842-2229

Biotechnology Information Center
National Agricultural Library
10301 Baltimore Blvd.
Beltsville, MD 20705
phone 301-344-3218

Industrial Biotechnology Association
1625 K Street, NW
Suite 1100
Washington, D.C. 20006
phone 202-857-0244

Office of Recombinant DNA Activities (Copies of the NIH guidelines may be obtained by
National Institutes of Health writing this office)
Building 31, Room 4B11
Bethesda, MD 20892

Tissue Culture Association
8815 Centre Park Dr. Suite 210
Columbia, MD 21045
phone 301-992-0946

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This 8 page pamphlet describes the impact of biotechnology on medicine and how it might be used to combat diseases.

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This coffee table book gives a good overview of what biotechnology is. It is well illustrated.

Arber, Werner, Karl Illmensee, W. James Peacock and Peter Starlinger. ed. 1984 Genetic Manipulation: Impact on Man and Society. Cambridge University Press, Cambridge.

This 250 page book is the result of a symposium of the same name.

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This 84 page publication outlines the scientific and technological developments in Biotechnology to the date of publication. The implications for government policy is also outlined.

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Coombs, J. and Y.R. Alston. 1990. The Biotechnology Directory: 1991: Products, Companies, Research and Organizations. M Stockton Press, NY.

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This is a special issue of The Ohio Journal of Science and resulted from two symposia that were held at the 1986 annual meeting of The Ohio Academy of Science. One symposium dealt with the economic aspects of biotechnology and the other dealt with educational aspect of biotechnology.

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This book traces the transformation of an academic science into technology. It discusses how biotechnology has helped transform the relationship between the university and industry and business.

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Ouellette, Robert P. and Paul N. Cheremisinoff. 1985. Essential of Biotechnology. Technomic Publishing Co., Inc., Lancaster.

This 226 page book gives an overview of biotechnology. It has numerous references.

Phillips, Marshall, Sharon P. Shoemaker, Roger D. Middlekauff and Raphael M. Ottenbrite, ed. 1988. The Impact of Chemistry on Biotechnology: Multidisciplinary Discussions. American Chemical Society, Washington, D.C.

This book is the result of a symposium of the same name. The purpose of the book is to demonstrate the relationship between chemistry and the advances in biotechnology.

Pines, Maya. 1984. The New Human Genetics: How Gene Splicing Helps Researchers Fight Inherited Disease. U.S. Dept. of Health and Human Resources, Washington.

This is an excellent 61 page booklet that describes how biotechnology can impact the treatment of genetic disorders in humans. NIH Publication NO. 84-662.

Postgate, John. 1986. Microbes and Man. Penguin Books, New York.

This book deals with how microbes impact man and how microbes can be used.

Präve, Paul, Uwe Faust, Wolfgang Sattig, and Dieter A. Sukatsch, ed. 1989. Basic Biotechnology: A Student's Guide. VCH Publishers, New York.

This is an excellent book. It gives an overview of biotechnology and it should be useful to students.

Prentis, Steve. 1984. Biotechnology: A New Industrial Revolution. George Braziller, Inc., NY.

An excellent intro to biotechnology.

Rasmussen, Alison and Robert H. Matheson III. 1990. A Sourcebook of Biotechnology Activities. National Association of Biology Teachers and North Carolina Biotechnology Center, Reston.

A publication for teachers which has been field tested in classrooms. Each activity has teacher page, and student pages. This publication is well done and should be very useful to teachers. The publication may be obtained from NABT.

Rosenfield, Israel, Edward Ziff and Borin Van Loon,. 1983. DNA for Beginners. Writers and Readers Publishing Corp. Ltd., London.

An excellent book written in a comic book format. This should be on the shelves of school libraries.

Sasson, Albert. 1988. Biotechnologies and Development. UNESCO Technical Centre for Agricultural and Rural Cooperation (CTA), Paris.

This book deals with the promise of biotechnologies for developing countries as well as the difficulties and constraints that these countries face.

Sattelle, David. 1990. Biotechnology in Perspective. Industrial Biotechnology Association, Washington.

This excellent 46 page booklet gives and overview of biotechnology. It has discussion questions and review questions for students.

Smith, George P. II. 1989. The New Biology: Law Ethics, and Biotechnology. Plenum Press, N.Y.

This book explores that challenges and opportunities that today's biology presents society.

St. Louis Mathematics and Science Education Center. 1989. Biotechnology Unit for Secondary Students. St. Louis Math and Science Education Center, St. Louis.

This is a publication for the classroom that has been teacher developed and tested. It contains teacher notes as well as pages which may be copied for students. This publication may be obtained from the Mathematics and Science Education Center located at U.M. - St. Louis, 8001 Natural Bridge Rd., St. Louis, MO 63121.

Subcommittee on Investigations and Oversight of the Committee on Science and Technology of the House of Representatives, 99th Congress, second session. 1986. Issues in the Federal Regulation of Biotechnology: From Research to release. U.S. Government Printing Office, Washington, D.C.

This report looks at issues involved with release of organisms and gives recommendation as to how the government should deal with these issues.

Sylvester, Edward J. and Lynn C. Klotz. 1983. The Gene Age: Genetic Engineering and The Next Industrial Revolution. Charles Scribner's Sons, New York.

This book gives a basic description of genetic engineering and how this technology will impact business and industry.

The Presidential Council of Competitiveness (Quayle, Dan, Chairman). 1991. Report on National Biotechnology Policy. Dept. of Commerce, Washington.

This report gives recommendations to the administration on how the US may remain competitive in the biotechnology industry.

U.S. Congress, Office of Technology Assessment. 1987. New Developments in Biotechnology: (v.1) Ownership of Human Tissues and Cells-Special Report, OTA-BA-337. U.S. Government Printing Office, Washington, D.C.

OTA analyzes the economic, legal and ethical rights of the sources of human tissues and those of researchers that develop these biological materials.

U.S. Congress, Office of Technology Assessment. 1988. New Developments in Biotechnology: (v.4) U.S. Investment in Biotechnology-Special Report, OTA-BA-360. U.S. Government Printing Office, Washington, D.C.

The report describes the levels and types of investment that were being made as of 1988. Ten major issues that affect investment were identified. This publication also identifies companies involved in biotechnology.

Vasil, Indra K. 1987. Biotechnology: Perspectives, Policies and Issues. University of Florida Press, Gainesville.

This book was based on a symposium of the same name. It dealt with advances of biotechnology in various field, and industrial perspectives and commercialization issues.

Watson, James D. 1968. Double Helix: Being a Personal Account of the Discovery of the Structure of DNA. Macmillian Publishing Company, Atheneum, NY.

This book is a must for the school library.

Woodhead, Avril D. and Benjamin J. Barnhart. 1988. Biotechnology and The Human Genome: Innovations and Impact. Plenum Press, NY.

This 175 page book is the result of a workshop of the same name held for science writers. The purpose was to give writers a first hand knowledge at the human gnome project.

Zimmerman, Burke K. 1984. Biofuture: Confronting the Genetic Era. Plenum Press, NY.

This book is very readable. It outlines historical development of genetic engineering and discusses the ethical issues associated with the power that knowledge of molecular genetics has given us.

Biotechnology in Food Production

Angold, Roger, Gordon Beech and John Taggart. 1989. Food Biotechnology. Cambridge University Press.

The purpose of this 171 page book is to demonstrate how biotechnology is being used by the food industry.

Banwart, George J. 1989. Basic Food Microbiology. Van Nostrand Reinhold, N.Y.

This book contains a chapter on useful microbes.

Jay, James M. 1986. Modern Food Microbiology. 3rd ed. Van Nostrand Reinhold, New York.

This text describes the role of microbes in food production, food spoilage, food-borne pathogens, as well as food preservation techniques.

Lindsay, Robert C. and Brian J. Willis. 1989. Biotechnology Challenges for the Flavor and Food Industry. Elsevier Applied Science, New York.

This 170 page book is based on a symposium of the same name. It is written in such a way that students should find parts of it very useful.

Plucknett, Donald, Migel Smith, J.T. Williams and N. Murthi Anishetty. 1987. Gene Banks and the Work's Food. Princeton University Press, Princeton.

Genes Banks and their history are outlined. The potential for biotechnology techniques to utilize the genes in these banks is described.

Rogers, P.L. and G.H. Fleet. 1989. Biotechnology and The Food Industry. Gordon and Breach Science Publishers, NY.

An excellent overview of the ways in which biotechnology is impacting the food industry and opportunities for the use of biotechnology techniques in the future. This appears to be a book that would be very useful to students.

Tombs, M.P. 1990. Biotechnology in the Food Industry. open University Press, Milton Keynes.

This book gives an overview of how biotechnology is used in the food industry.

Biopolymer Synthesis

Doi, Yoshiharu. 1990. Microbial Polyesters. VCH Publishers, Inc., N.Y.

This 156 page book deals with the production of polyesters by bacteria. These products are of great interest to industry.

Tarchevsky, I.A. and G.N. Marchenko. 1991. Cellulose: Biosynthesis and Structure. Springer-Verlag, NY.

This book deals with the synthesis of cellulose in plants. There is some treatment of cellulose production by *Acetobacter*.

Nakas, James P. and Charles Hagedorn. 1990. Biotechnology of Plant Microbe Interactions. McGraw-Hill Publishing Co., New York.

This book attempts to provide an overview of biotechnology with regard to agronomically significant crops.

Plant Biotechnology

Austin, R.B., R.B. Flavell, I.E. Henson and H.J.B. Lowe. 1986. Molecular Biology and Crop Improvement: A Case Study of Wheat, Oilseed Rape, and Faba Beans. Cambridge University Press, New York.

This book is a report on the opportunities for the application of Molecular Biology to Crop Improvement.

Bajaj, Y.P.S. 1986. Biotechnology in Agriculture and Forestry I: Trees I. Springer-Verlag, New York.

This book describes techniques that may be used to manipulate trees *in vitro*. Techniques for several species of trees are given.

Conger, B.V., ed. 1981. Cloning Agricultural Plants via *in vitro* Techniques. CRC Press, Boca Raton.

This publication describes techniques and media to culture plants of agricultural importance.

Gleba, Y.Y. and K.M. Sytnik. 1984. Protoplast Fusion: Genetic Engineering in Higher Plants. Springer-Verlag, NY.

This book has a number of excellent illustrations and should be useful for learning about plant cell fusion.

Lynch, J.M. 1983. Soil Biotechnology: Microbiological Factors in Crop Productivity. Blackwell Scientific Publications, Oxford.

This 191 page book deals with how microbial activity might be manipulated to improve agricultural and horticultural productivity.

Mantell, S.H., J.A. Matthews and R.A. McKee. 1985. Principles of Plant Biotechnology: An Introduction to Genetic Engineering in Plants, Blackwell Scientific Publications, Boston.

This book deals with plant biotechnology as it relates to crop improvement.

Moore, Thomas C. 1989. Biochemistry and Physiology of Plant Hormones 2nd ed. Springer-Verlag, NY.

This book should be of use to those doing plant tissue culture.

Neumann, K.-H., W. Barz and E. Reinhard. 1985. Primary and Secondary Metabolism of Plant Cell Cultures. Springer-Verlag, New York.

The book is the result of a symposium of the same name. It covers potential biotechnological applications of plant cell cultures.

Oliver, Stephen and Terence Brown. 1985. Microbial Extrachromosomal Genetics. American Society for Microbiology, Washington.

This small book includes a discussion of mitochondrial and chloroplast genetics as well as a discussion of yeast plasmids.

Pierik, R.L.M. 1987. *In vitro* Culture of Higher Plants. Martinus Nijhoff Publishers, Boston.

This book describes basic tissue and cell culture techniques.

Robins, R.J. and M.J.C. Rhodes. ed. 1988. Manipulating Secondary Metabolism in Culture. Cambridge UniversityPress, NY.

This book surveys the utilization of biotechnology to produce useful products from plant cell culture.

Wickner, Reed B., Alan Hinnebusch, Alan M. Lambowitz, I.C. Gunsalus and Alexander Hollaender. 1986. Extrachromosomal Elements in Lower Eukaryotes. Plenum Press, NY.

This book deals with plasmids of yeast, fungi, protozoa, slime molds, and algae. It also deals with mitochondria and chloroplast DNA.

Yeoman, M.M. 1986. Plant Cell Culture Technology. Blackwell Scientific Publications, Boston.

In addition to the basics, this book has a chapter on industrial applications and one on genetic manipulation.

Enzyme Expression and Production

Darnell, James, Harvey Lodish and David Baltimore. 1990. Molecular Cell Biology. 2nd ed. Scientific American Books.

This is a well written comprehensive, integrated account of life processes at the molecular and cellular level.

Dawson, Jeffrey R., Edward Drexler, Paula K. Haddon, Joye F. Jones, Joseph McInerney and Jean P. Thibault. 1986. Immunology and Human Health. BSCS and The Foundation for Blood Research, Scarborough and Colorado Springs.

This publication is meant for teachers to use in the classroom. It is well thought out and it was field tested before publication.

Erickson, Larry E. and Daniel Yee-chak, Fung. ed. 1988. Handbook on anaerobic fermentations. Marcel Dekker Inc., New York.

This book deals with biotechnology and biochemical engineering associated with anaerobic fermentations.

Freifelder, David. 1987. Molecular Biology. 2nd ed. Jones and Bartlett, Inc., Boston.

This is a well written introduction to molecular biology.

Halpern, M.G., editor. 1981. Industrial Enzymes from Microbial Sources: Recent Advances. (Chemical Technology Review Ser.: No. 186). Noyes Data Corporation, Park Ridge, New Jersey.

This book describes methods of producing various enzymes from microbes as well as extraction methods.

Harris, T.J.R., ed. 1990. Protein Production by Biotechnology. Elsevier Applied Science, New York.

The authors in this volume discuss various methods by which protein might be produced. This includes bacteria, yeast, mammalian cells, fungi as well as whole plants and animals.

Hollenberg, C.P. and H. Sahm. 1987. Microbial Genetic Engineering and Enzyme Technology. Gustav Fischer, Verlag New York.

This 142 page book is divided into two sections. The first is entitled "Microbial Genetic Engineering" and contains 7 papers and the second is entitled "Enzyme technology" and contains 8 papers.

Laskin, Allen I. ed. 1985. Enzymes and Immobilized cells in Biotechnology. Benjamin/Cummings Publishing Co., Menlo Park.

This book deals with the way in which Immobilized Enzyme can be used in the Biotechnology Industry by giving specific examples.

Moody, Peter C.E. and Anthony J. Wilkinson. 1990. Protein Engineering. IRL Press, New York.

This 85 page book gives an overview of protein engineering. The reader needs a solid foundation in math.

Narang, Saran A., ed. 1990. Protein Engineering: Approaches to the Manipulation of Protein Folding. Butterworths, Boston.

This book gives an overview of how protein structure may be studied and manipulated in order to better understand protein folding.

Priest, Fergus G. 1984. Extracellular Enzymes. American Society for Microbiology, Washington.

This 79 page book describes enzyme production by cells, enzyme regulation, and how this enzyme production may be used for commercial purposes.

Stein, Stanley. 1990. Fundamentals of Protein Biotechnology. Marcel Dekker, Inc., NY.

This book focuses on protein structure biological properties, purification, analysis, synthesis and production.

Stryer, Lubert. 1988. Biochemistry. 3rd ed. W.H. Freeman and Co., New York.

This is an excellent biochemistry book and should be included in the reference materials in high school libraries.

Altering Genetic Characteristics

Alberts, Bruce, Dennis Bray, Julian Lewis, Martin Raff, Keith Roberts and James D. Watson. 1989. Molecular Biology of the Cell. 2nd ed. Garland Publishing, Inc., New York.

An excellent reference which should be included in the high school library.

Anonymous. Genetic Engineering: A Natural Science. Monsanto Co., St. Louis.

A well illustrated and written 24 page brochure that explains what genetic engineering is and how it may be used.

Barr, Philip J., Anthony J. Brake and Pablo Valenzuela. 1989. Yeast Genetic Engineering. Butterworths, Boston.

This book attempts to intergrate academic and applied research involving yeast. This book will be of use to a few secondary students.

Boyd, Robert. 1988. General Microbiology Times Mirror/Mosby College Publishing.

This is a good microbiology text that can serve as a reference for students.

Broda, Paul. 1979. Plasmids. W.H. Freeman and Co., San Francisco.

This is an excellent reference for secondary students.

Campbell, I. and J.H. Duffus. 1988. Yeast: A Practical Approach. IRL Press, Washington, D.C.

This is a good reference for those working with yeast.

Denniston, K.J. and L.W. Enquist. 1981. Recombinant DNA (Benchmark Papers in Microbiology Vol. 15). Dowden Hutchinson and Ross, Inc., Stroudsburg.

This book is a collection of 35 reprinted journal articles. The collection is the attempt of the editors to define some of the critical work that helped create recombinant DNA technology as we know it today.

Doelle, H.W. and C.-G. Hed-en. ed. 1986. Applied Microbiology. D. Reidel Publishing Co., Boston.

This 192 page book deals with potentials and problems of microbial conversion technology.

Drlica, Karl and Monica Riley., ed. 1990. The Bacterial Chromosome. American Society for Microbiology. Washington, D.C.

This 469 page book contains 39 paper in 8 sections including the prologue and epilogue. The genetic map of *E. coli* and the map of *Bacillus subtilis* 168 are given. The book gives an overview of the chromosome including the configuration of DNA, replication, recombination and responses to stress.

Esser, K., V. Kück, C. Lang-Hinrichs, P. Lemke, H.D. Osienwacz, V. Stahl and P. Tudzynski. 1986. Plasmids of Eukaryotes. Springer-Verlag, New York.

This 124 page book gives an overview of plasmids in eukaryotes. This book does give historical and experimental background which helps make it even more useful to students.

Freifelder, David. 1987. Microbial Genetics. Jones and Bartlett Publishers, Inc., Boston.

This is an excellent textbook. It should be useful to high school students.

Freifelder, David. 1987. Molecular Biology. 2nd ed. Jones and Bartlett, Inc., Boston.

This is a well written introduction to molecular biology.

Glover, D.M., editor. 1985. DNA Cloning, Vol. I. and II (The Practical Approach Ser.). IRL Press, Oxford; Washington DC.

These two volumes are well written publications with description of a number of important techniques.

Hames, B.D. and D. M. Glover. 1990. Gene Rearrangement. IRL Press, Oxford.

This 154 page book covers DNA amplification in eukaryotes, antigenic variation in trypanasomes, and the molecular basis of genomic rearrangement in prokaryotes.

Hardy, Kimber. 1986. Bacterial Plasmids. 2nd ed. American Society for Microbiology, Washington.

This 114 page book gives an overview of what plasmids are, the types that are found and how they may be used in biotechnology. This is an excellent reference for students.

Hendrix, Roger W., Jeffrey Roberts, Franklin Stahl, and Robert A. Weisberg. 1983. Lambda II. Cold Spring Harbor Laboratory, Cold Spring Harbor.

This book gives a thorough description of the lambda bacteriophage.

Kingsman, S.M. and A.J. Kingsman. 1988. Genetic Engineering: An Introduction to Gene Analysis and Exploitation in Eukaryotes. Blackwell Scientific Publications, Cambridge, Massachusetts.

This book gives an introduction to the eukaryote genome and to genetic engineering. It describes how genetic engineering is being used in eukaryotes and how it might be used. The book has an extensive reference list It is well written and should be of use to students interested in this area.

Kirsop, B.E. and C.P. Kurtzman., ed. 1988. Yeasts: Living Resources for Biotechnology. Cambridge University Press, New York.

This book provides information on sources of yeast, storage, identification and other information that is useful to those that are working with yeast.

Kornberg, Arthur. 1991. DNA Replication. 2nd ed. W. H. Freeman and Company, New York.

This book goes into great detail and should not be viewed by students reading about DNA for the first time.

Lewin, Benjamin. 1990. Genes IV. Oxford University Press, New York.

This is a well written book on the molecular biology of the gene. It should prove to be of use to students and teachers.

Micklos, David A. and Greg A. Freyer. 1990. DNA Science: A First Course in Recombinant DNA Technology. Carolina Biological Supply Co. and Cold Spring Harbor Laboratory Press, Burlington and Cold Spring Harbor.

If not used in class, this book ought to be in every high school library and on the references shelf of every high school biology teacher. The authors of this book have done much to make DNA technology at the high school level a reality. This book is very readable by students.

Neidhardt, Frederick C., ed. 1987. *Escherichia coli* and *Salmonella Typhimurium*: Cellular and Molecular Biology, Volumes I and II. American Society for Microbiology, Washington.

These two volumes bring together description of our existing body of knowledge on the molecular and cellular biology of these two organisms. Over 130 experts were involved in the writing of these two volumes.

Old, R.W. and S.B. Primrose. 1985. Principles of Gene Manipulation. 3rd ed. Blackwell Scientific Publications, Oxford.

This book describes the basics of genetic engineering and it has an extensive reference section. This is an excellent book.

Oliver, Stephen and Terence Brown. 1985. Microbial Extrachromosomal Genetics. American Society for Microbiology, Washington.

This small book includes a discussion of mitochondrial and chloroplast genetics as well as a discussion of yeast plasmids.

Setlow, J.K., editor. 1979. Genetic Engineering: Principles & Methods, Vol. 1-12. Plenum Publishing Corporation, NY.

This is a series of publications that describe advancements in genetic engineering.

Strathern, Jeffrey N., Elizabeth W. Jones, and James Broach, ed. 1982. The Molecular Biology of Yeast *Saccharomyces*: Life Cycle and Inheritance. Cold Spring Harbor Laboratory, Cold Spring Harbor.

Strathern, Jeffrey N., Elizabeth W. Jones, and James R. Broach, ed. 9181. The Molecular Biology of Yeast *Saccharomyces*: Metabolism and Gene Expression. Cold Spring Harbor Laboratory, Cold Spring Harbor.

The purpose of these two volumes was to summarize the knowledge about *Saccharomyces* and to give direction for research in the 1980's. There are extensive references given for the work that was done on yeast before 1980.

Walton, E.F. and G.T. Yarranton. 1989. Molecular and Cell Biology of yeasts. Blackie and Van Nostrand Reinhold, Glasglow and NY.

Although an excellent book, most students would find it to be very difficult reading.

Warr, J. Roger. 1984. Genetic Engineering in Higher Organisms: The Institute of Biology's Studies in Biology No. 162. Edward Arnold, Baltimore.

This 58 page book deals with mammalian and plant cells and their uses. As with the other books in this series, this is an excellent book and should be in the secondary school library.

Watson, James D., Nancy Hopkins, Jeffrey Roberts, Joan Argetsinger Steitz, and Alan M. Weiner. 1987. Molecular Biology of the Gene. 4th ed. The Benjamin/Cummings publishing Co., Inc., Menlo Park

This two volume edition is a continuation of the classic first edition which was written by James Watson.

Watson, James, John Tooze, David T. Kurtz. 1983. Recombinant DNA: A Short Course. W.H. Freeman and Co., New York.

This book ought to be in every high school library.

Wickner, Reed B., Alan Hinnebusch, Alan M. Lambowitz, I.C. Gunsalus and Alexander Hollaender. 1986. Extrachromosomal Elements in Lower Eukaryotes. Plenum Press, NY.

This book deals with plasmids of yeast, fungi, protozoa, slime molds, and algae. It also deals with mitochondria and chloroplast DNA.

Applications of DNA Analysis

Alberts, Bruce, Dennis Bray, Julian Lewis, Martin Raff, Keith Roberts and James D. Watson. 1989. Molecular Biology of the Cell. 2nd ed. Garland Publishing, Inc., New York.

An excellent reference which should be included in the high school library.

Cooper, Geoffrey M. 1990. Oncogenes. Jones and Bartlett Publishers, Boston.

This book describes the role of genes in the development of cancer. It also discusses the role of these genes in the cell.

Freifelder, David. 1987. Microbial Genetics. Jones and Bartlett Publishers, Inc., Boston.

This is an excellent textbook. It should be useful to high school students.

Freifelder, David. 1987. Molecular Biology. 2nd ed. Jones and Bartlett, Inc., Boston.

This is a well written introduction to molecular biology.

Glover, D.M., editor. 1985. DNA Cloning, Vol. I. and II (The Practical Approach Ser.). IRL Press, Oxford; Washington DC.

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Hollenberg, C.P. and H. Sahm. 1987. Microbial Genetic Engineering and Enzyme Technology. Gustav Fischer, Verlag New York.

This 142 page book is divided into two sections. The first is entitled "Microbial Genetic Engineering" and contains 7 papers and the second is entitled "Enzyme technology" and contains 8 papers.

Keller, George H. and Mark M. Manak. 1989. DNA Probes. M. Stockton Press, New York.

This book describes how to make and use DNA probes.

Kingsman, S.M. and A.J. Kingsman. 1988. Genetic Engineering: An Introduction to Gene Analysis and Exploitation in Eukaryotes. Blackwell Scientific Publications, Cambridge, Massachusetts.

This book gives an introduction to the eukaryote genome and to genetic engineering. It describes how genetic engineering is being used in eukaryotes and how it might be used. The book has an extensive reference list It is well written and should be of use to students interested in this area.

Larson, Jean A. 1991. BST - Bovine Somatotropin/Growth Hormone: January 1986 - December 1990. (Quick Bibliography Series) National Agricultural Library, Beltsville.

This bibliography contains 259 citations and is available from the Animal Welfare Information.

Lewin, Benjamin. 1990. Genes IV. Oxford University Press, New York.

This is a well written book on the molecular biology of the gene. It should prove to be of use to students and teachers.

Micklos, David A. and Greg A. Freyer. 1990. DNA Science: A First Course in Recombinant DNA Technology. Carolina Biological Supply Co. and Cold Spring Harbor Laboratory Press, Burlington and Cold Spring Harbor.

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Old, R.W. and S.B. Primrose. 1985. Principles of Gene Manipulation. 3rd ed. Blackwell Scientific Publications, Oxford.

This book describes the basics of genetic engineering and it has an extensive reference section. This is an excellent book.

Roberts, D.W., and R.R. Grandos. 1989. Biotechnology, Biological Pesticides and Novel Plant-Pest resistance for Insect pest management. Insect Pathology Resource Center at Boyce Thompson Institute for Plant research, Ithaca.

This monograph was the result of a conference of the same title held in 1988. This monograph serves to demonstrate ways in which biotechnology can be used to develop microbial pesticides. These new methods could have significant benefits for the environment. This monograph is available from the Boyce Thompson Institute for Plant Pathology at Cornell University. Ithaca, New York, 14853.

Rodriquez, Raymond L. and David T. Denhardt. ed. 1988. Vectors: A Survey of Molecular Cloning Vectors and Their Uses. Butterworths, Boston.

This book describes vectors of various hosts and the application of those vectors.

Seetharam, Ramnath and Satish K. Sharma. 1991. Purification and Analysis of Recombinant Proteins. Marcel Dekker. Inc., NY.

This book is concerned with bioprocess technology. It looks at various methods of protein purification an how to engineer sites to assist protein purification and how to meet the challenges of protein purification.

Setlow, J.K., editor. 1979. Genetic Engineering: Principles & Methods, Vol. 1-12. Plenum Publishing Corporation, NY.

This is a series of publications that describe advancements in genetic engineering.

Watson, James, John Tooze, David T. Kurtz. 1983. Recombinant DNA: A Short Course. W.H. Freeman and Co., New York.

This book ought to be in every high school library.

Protein Analysis

Hames, B.D. and D. Rickwood., ed. 1990. Gel Electrophoresis of Proteins: A Practical Approach. 2nd ed. IRL Press, New York.

This book covers the various techniques by which gel electrophoresis may be used to separate proteins. It is well written and should prove to be a useful reference.

Moody, Peter C.E. and Anthony J. Wilkinson. 1990. Protein Engineering. IRL Press, New York.

This 85 page book gives an overview of protein engineering. The reader needs a solid foundation in math.

Narang, Saran A., ed. 1990. Protein Engineering: Approaches to the Manipulation of Protein Folding. Butterworths, Boston.

This book gives an overview of how protein structure may be studied and manipulated in order to better understand protein folding.

Stein, Stanley. 1990. Fundamentals of Protein Biotechnology. Marcel Dekker, Inc., NY.

This book focuses on protein structure biological properties, purification, analysis, synthesis and production.

Industrial and Environmental Biotechnology

Committee of the Introduction of Genetically Engineered Organisms Into the Environment. 1987. Introduction of recombinant DNA-Engineered Organisms Into the Environment: Key Issues. National Academy Press, Washington.

This small publication gives an overview of a key issues in biotechnology and the conclusions of the subcommittee which dealt with those issues.

Committee on Scientific Evaluation of the Introduction of Genetically Modified Microorganisms and Plants into the Environment. 1989. Field Testing Genetically Modified Organisms: Framework for Decisions. National Academy Press, Washington, D.C.

The purpose of this book was to define the scientific information and issues surrounding the topic, and to develop recommendation for policy makers to consider.

Demain, Arnold L. and Nadine A. Solomon. 1985. Biology of Industrial Microorganisms. The Benjamin/Cummings Publishing Co., Inc., Menlo Park.

This book covers basic information on a number of organisms, including: *E. coli*, bacilli, actinomycetes, fungi and yeasts.

Demain, Arnold L. and Nadine A. Solomon, ed. 1986. Manual of Industrial Microbiology and Biotechnology. American Society for Microbiology, Washington.

This book gives outlines the biological and engineering methodology required to develop a successful industrial process form the isolation of the culture to the isolation of product.

Ehrlich, Henry L. and Corale L. Brierley. 1990. Microbial Mineral Recovery. McGraw-Hill Publishing Co., New York.

This book gives an overview of how bacteria may be used to collect minerals and how the may be used to clean coal and petroleum.

Erickson, Larry E. and Daniel Yee-chak, Fung. ed. 1988. Handbook on anaerobic fermentations. Marcel Dekker Inc., New York.

This book deals with biotechnology and biochemical engineering associated with anaerobic fermentations.

Greenbaum, Elias and Charles E. Wyman. 1991. Twelfth Symposium on Biotechnology for Fuels and Chemicals. Applied Biochem. and Biotech. Vol. 28 and 29. Humana Press, Clifton, N.J.

In addition to production of fuels and products such as enzyme, this volume also deals with environmental issues.

Gross, Cynthia. 1988. The New Biotechnology: Putting Microbes to Work. Lerner Publication Co., Minneapolis, MN.

This easy to read 96 page book gives an overview of biotechnology and it relates to a number of fields. This book should be in the school library.

Hartmeier, Winfried. 1988. Immobilized Biocatalysts: an introduction. Springer-Verlag, New York.

In addition to explaining principles and methods, this book also describes experiments with immobilized biocatalysts.

Healy, Bernadine P. (chair). 1988. New Developments in Biotechnology: Field-Testing Engineered Organisms: Genetic and Ecological Issues. Technomic Publishing Co., Lancaster.

This publication is developed to guide congress and other public policy decisions makers.

Hollaender, A., editor. 1981. Trends in the Biology of Fermentations for Fuels & Chemicals. (Basic Life Science Ser.: Vol. 18). Plenum Publishing Corporation, NY.

This book will not be of interest to most students. However it may prove to be helpful to those that get deeply involved in microbial fermentation.

Horan, N.J. 1990. Biological Wastewater Treatment Systems: Theory and operation. John Wiley and Sons, New York.

This book deals with concepts of microbial growth and reactor engineering which deal with wastewater treatment systems.

Klingmüller, Walter, ed. 1988. Risk Assessment for Deliberate Releases: The Possible Impact of Genetically Engineered Microorganisms on The Environment. Springer-Verlag, New York.

This 193 page book is the result of a European symposium of the same name. Twenty-one papers make up this volume which deals with topics on developing model systems to regulation of release.

Labeda, David P., ed. 1990. Isolation of Biotechnological Organisms from Nature. McGraw-Hill Publishing Company, N.Y.

This book discusses a wide variety of organism of potential sources for Biotechnology.

Leong, Sally A. and Randy M. Berka, ed. 1991. Molecular Industrial Mycology: Systems and Applications for Filamentous Fungi. Marcel Dekker, Inc., New York.

This book describes how fungi can be manipulated at the molecular level and examples of how fungi are used are given.

Levin, Morris and Harlee S. Strauss, ed. 1991. Risk Assessment in Genetic Engineering. McGraw-Hill, Inc., New York.

This volume contains contributions from experts in many areas and thus takes a comprehensive look at the issue.

Lewis, Chris. 1983. Biological Fuels. Edward Arnold, London.

This 59 page publication is one of the institute of biology's studies in biology series (#153). It should be of use to student interested in biomass conversion.

Mizrahi, Avshalom., ed. 1989. Advances in Biotechnological Processes Vol. 12: Biological Waste Treatment. Alan R. Liss, Inc. New York.

This book is part of a series dealing with biotechnology.

Moo-Young, M., J. Lamptey, B. Glick and H. Bungay., ed. 1987. Biomass Conversion Technology: Principles and Practice. Pergamon Press, New York.

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A SAMPLING OF BIOTECHNOLOGY AND RELATED PERIODICALS

The addresses provided below are meant as sources of information for librarians and others interested in subscribing to these journals. The addresses are not provided for the solicitation of information.

Applied and Environmental Microbiology
American Society for Microbiology
1325 Massachusetts Ave. NW
Washington, D.C. 20005

Bioessays
The Company of Biologists Limited, UK
Bidder Building
140 Cowley Rd.
Cambridge, CB4 4DL, UK

Biotechniques
Eaton Publishing
154 E. Central St.
Natick, MA 01760

Biotechnology
Nature Publishing Co.
65 Bleecher St.
New York, N.Y. 10012-2467

Biotechnology Education
Pergamon Press Inc.
Maxwell House, Fairview Park
Elmsford, N.Y. 10523

Biotechnology Letters
Science and Technology Letters
P.O. Box 81, Northwood
Middlesex, HA6 3DN, England

Gene
Elsevier Science Publishers B.V.
Journals Dept.
P.O. Box 211
1000 AE Amsterdam
The Netherlands

Genetic Engineering News
Mary Ann Liebert Inc. Publishers
1651 Third Ave.
New York, N.Y. 10128

Journal of Bacteriology
American Society for Microbiology
1325 Massachusetts Ave. NW
Washington, D.C. 20005

Journal of Biotechnology
Elsevier Science Publishers
Journal Info. Center
655 Ave. of the Americas
New York, N.Y. 10010

Microbiological Reviews
American Society for Microbiology
1325 Massachusetts Ave. NW
Washington, D.C. 20005

Molecular Microbiology
Blackwell Scientific Publication Ltd.
Journal Subscription Dept.
Marston Bood Services
P.O. Box 87
Oxford OX2 ODT, UK

Nucleic Acids Research
Oxford University Press
P.O. Box Q
McLean, VA. 22101-0850.

Plant Cell, Tissue and Organ Culture
Kluwer Academic Publishers
P.O. Box 17
3300 AA Dordrecht
The Netherlands

Plasmid
Academic Press
1 East First St.
Duluth, MN 55802

Process Biochemistry
Turret Group
P.O. Box 64
Rickmansworth, Herts, UK WD3 1CN

Science
American Association for the
Advancement of Science
1333 H Street NW
Washington, D.C. 20005

Trends in Biotechnology
Elsevier Science Publishers, Ltd.
655 Ave. of the Americas
New York, N.Y. 10010

Trends in Genetics
Elsevier Science Publishers, Ltd.
655 Ave of the Americas
New York, N.Y. 10010

ANNOTATED LIST OF BIOTECHNOLOGY MATERIALS AND EQUIPMENT SUPPLIERS

BIOTECHNOLOGY MATERIALS CODE

A agarose

AB antibodies

AE agarose electrophoresis equipment

AN antibiotics

AT animal tissue culture media

B Bacteriological media

BC bacterial strains

BM Biotechnology experiment modules

BU Buffers

CH chromatography equipment

CS camera system

CT centrifuge tubes

CU culture tubes

D DNA

DP DNA probes

DS DNA size standards

FI film

G gloves

GO goggles

LG laboratory glassware

M microcentrifuge

MC microcentrifuge tubes

ME DNA modifying enzymes

MP microliter pipets

MR microcentrifuge tube racks

MS membranes for Southern blots

MT microliter piper tips

NR Non-radioactive DNA labeling kit

P pipets

PA protein assay kit

PC precast polyacrylamide gels

PD petri dishes

PE polyacrylamide electrophoresis equipment

PP pipet pump

PS protein size standards

PT plant tissue culture media

R reagents

RE restriction enzymes

S stains

S sterilization indicators

TC tissue culture apparatus

For a more complete listing of companies and products relating to biotechnology see the Biotechnology Products and Instruments issue of *Science*. This special issue is published annually by the American Association for the Advancement of Science. This issue may be found at libraries subscribing to *Science*.

Amresco
30175 Solon Industrial Pkwy.
Solon, OH 44139

AB, AE, BU, R, S

Bellco Glass Inc.
340 Edrudo Rd.
P.O. Box B
Vineland, NJ 08360-0117
phone 1-800-222-0227

CT, CU, P, TC

Bio-Rad Laboratories
220 Maple Ave. P.O. Box 708
Rockville Centre, NY 11571
phone 1-800-645-3227

A, AE, CH, DS MS, MT PA, PE, PS

Boehringer Mannheim Biochemicals
Biochemicals Division
P.O. Box 50816
Indianapolis, IN 46250
phone 1-800-428-5433

A, AN, DS, ME, NR, RE

Calbiochem Corporation
P.O. Box 12087
San Diego, CA 92112-4180
phone 1-800-854-3417

AN, D, R, RE

Carolina Biological Supply Company
Main Office and Laboratories
Burlington, NC 27215
phone 1-800-334-5551

A, AE, AN, AT, B, BC, BM, CS, CT, CU, D, DS, FI, G, GO, M, MC, ME, MP, MR, MT, P, PD, PT, R, TC

(Serves as the retail outlet for the Cold Spring Harbor developed "DNA Science: A First Course in Recombinant DNA Technology")

C-Bio Management Company
Cell Culture Laboratory Division
4400 Emery Industrial Pkwy.
Cleveland, OH 44128
phone 1-216-765-8858

AT, R

Edvotek, Inc.
P.O. Box 1232
West Bethesda, MD 20827-1232
phone 1-800-EDVTEK or 1-301-252-5990

A, AE, B, BM, D, G, GO, M, MT, R, RE

Fotodyne Inc.
16700 West Victor Rd.
New Berlin, WI 553151-4131

AE, CS, FI, G, M, MC, MP, MR, MT

FMC BIOProducts
5 Maple St.
Rockland, ME 04841
phone 1-800-341-1574

A, NR (considered to have one of the best agarose products for biotechnology use)

Gibco/BRL
8400 Helgerman Court
Gaithersburg, MD 20877
phone 301-840-8000

A, AE, AN, AT, D, DS, ME, PT, R, RE

Hoefer Scientific Instruments
645 Minnesota St.
Box 77387
San Francisco, CA 94107
phone 1-800-227-4750

AE, CS, M, MC, MS, PE, PS ,R

International Biotechnologies Inc.
P.O. Box 9558
New Haven, CT 06535
phone 1-800-243-2555

AE, AN, BC, CS, DS, M, ME, MS, R, RE

Isolab, Inc.
P.O. Drawer 4350
Akron, OH 44321
phone 1-216-825-4525

PL, PE

Modern Biology, Inc.
P.O. Box 97
Dayton, Ind. 47941-0097

A, AE, B, BC, BM, BU, CH, CU, D, DS, PD, R, RE, S

New England Biolabs, Inc.
32 Tozer Rd.
Beverly, MA 01915-5510
phone: 1-617-927-5054

D, DP, DS, ME, RE

North American Science Associates, Inc.
2261 Tracy Rd.
Northwood, OH 43819
phone 1-419-666-9455

SI

Oncogene Science, Inc.
160 Charles Lindbergh Dr.
Uniondale, NY 11553
phone 1-516-222-0023

AB, DP

Perfector Scientific
P.O. Box 91
Atascada, CA 93423

LT, MC, MR, MT, PP

PGC Scientifics Corporation
P.O. Box 7277
Gaithersberg, MD 20898-7277

MC, PD, PP

Promega Corp.
2800 Woods Hollow Rd.
Madison, WI 53711
phone 1-800-356-9526

D, DP, ME, R, RE

Rainin Instruments Co.
Mack Rd.
Woburn, MA 01810
phone 1-617-935-3050

MP, MT

Stratagene Cloning Systems
11099 North Torrey Pines Rd.
La Jolla, CA 92037
phone 1-800-424-5444

AE, BC, D, ME, R, RE

Sigma Chemical Co.
P.O. Box 14508
St. Louis, MO 63178
phone 1-800-325-3010

AN, D, ME, PT, RE, TC

Tekmar Company
P.O. Box 371856
Cincinnati, OH 45222
phone 1-513-761-0633

P, PD, TC

United States Biochemical Corp.
P.O. Box 22400
Cleveland, OH 44122
phone 1-800-321-9322

D, ME, NR, R, RE

Variety Glass, Inc.
201-215 Foster Ave.
Cambridge, OH 43725
phone 1-614-432-3643

LG

Ward's Natural Science Establishment, Inc.
5100 West Henrietta Rd.
P.O. Box 92912
Rochester, NY 14692-9012
phone 1-800-962-2660

AT, CT, CU, PD, PT, R, TC

West Coast Scientific, Inc.
22542 Barrington Court
Hayward, CA 94545
phone 1-800-367-8462

AE, M, MC, MP, MR, MS, MT,
PE

Zaxis Inc.
Gel Trek Div. & Gel Teach Div.
1658 State Rd.
Cuyahoga Falls, OH 44223

BM, BU, PC, PE, R
(polyacrylamide eletrophoresis units
designed for the school setting)

STOCK CULTURES COLLECTIONS

Agricultural Research Service Culture Collection NRRL
Northern Regional Research Center
1915 N. University St.
Peoria Illinois 61604

The collection is comprised of strains primarily of bacteria, yeast, and fungi.

American Type Culture Collection
12301 Parklawn Dr.
Rockville, Maryland 20852-1776

ATCC has cultures of bacteria, bacteriophages, vectors, hosts, algae, protozoa, fungi, yeast, plant viruses, and antisera. There is have an educational rate for schools.

Bacillus Genetic Stock Center
The Ohio State University
Department of Biochemistry
484 W. 12th Ave.
Columbus, Ohio 43210

This stock center maintain strains of *Bacillus subtilis* as well as other strains of Bacillus.

Culture Collection of Algae
University of Texas at Austin
Austin, TX 78712

E. coli Genetic Stock Center
Dept. of Human Genetics
Yale University School of Medicine
New Haven, CT 06510

Fungal Genetics Stock Center FGSC
Department of Microbiology
University of Kansas Medical Center
Kansas City, Kansas 66103

Genetic Stocks of *Aspergillus nidulans*, *Neurospora* species and *Sordaria fimicola* are maintained.

Mid-American Drosophilia Stock Center
Department of Biology
Bowling Green State University
Bowling Green, Ohio 43403
Phone 419/372-2631

Yeast Genetic Stock Center
Department of Biophysics and Medical Physics
University of California
Berkley, California 94720

The stock center maintains 900 genetically defined strains of the yeast *Saccharomyces cerevisiae*. There is a fee of $10/culture with a minimum order of $25.

A SHORT SUMMARY OF NATIONAL INSTITUTES OF HEALTH (NIH) GUIDELINES

Purpose

The purpose of these NIH Guidelines is to specify practices for constructing and handling (i) recombinant DNA molecules and (ii) organisms and viruses containing recombinant DNA molecules.

Compliance

As a condition for NIH funding of recombinant DNA research, institutions must ensure that such research conducted at or sponsored by the institution, irrespective of the source of funding, shall comply with these Guidelines.

Voluntary Compliance

Individuals, corporations, and institutions not otherwise covered by the Guidelines are encouraged to do so by following the standards and procedures set forth in the Guidelines. For purposes of complying with the Guidelines, an individual intending to carry out research involving recombinant DNA is encouraged to affiliate with an institution that has an Institutional Biosafety Comm. (IBC) approved under the Guidelines.

Roles and Responsibilities

Safety in activities involving recombinant DNA depends on the individual conducting them. The Guidelines cannot anticipate every possible situation. Motivation and good judgment are the key essentials to protection of health and the environment.

Responsibility of the Institution

Each institution conducting or sponsoring recombinant DNA research covered by these Guidelines is responsible for ensuring that the research is carried out in full conformity with the provisions of the Guidelines. The institution shall establish and implement policies that provide for the safe conduct of recombinant DAN research and that endure compliance with the Guidelines.

The institution shall establish and IBC whose responsibilities need not be restricted to recombinant DNA.

Standard Practices and Training

The first principal of containment is a strict adherence to good microbiological practices. Consequently, all personnel directly or indirectly involved in experiments on recombinant DNAs must receive adequate instruction.

Any research group working with agents with a known or potential biohazard shall have an emergency plan which describes the procedures to be followed if an accident contaminates personnel or the environment. The Principal Investigator or Instructor PI must ensure that everyone in the laboratory is familiar with both the potential hazards of the work and the emergency plan.

Physical Containment Levels

The objective of physical containment is to confine organisms containing recombinant DNA molecules and thus to reduce the potential for exposure of the laboratory worker, persons outside of the laboratory, and the environment to organisms containing recombinant DNA molecules.

Combinations of laboratory practices, containment equipment, and special laboratory design can be made to achieve different levels of physical containment. Four levels of physical containment, which are designated as BL1, BL2, BL3, and BL4 are described. High School Laboratories are at the BL1 level.

Biosafety Level 1 is suitable for work involving agents of known or minimal potential hazard to laboratory personnel and the environment. The laboratory is not separated from the general traffic patters in the building. Work is generally conducted on open bench tops. Special containment equipment is not required or generally used. Laboratory personnel have specific training in the procedures conducted in the laboratory and are supervised by a scientist with general training in microbiology or related sciences.

Biosafety Level 2 is similar to Level 1 and is suitable for work involving agents of moderate potential hazard to personnel and the environment. It differs in that: (1) laboratory personnel have specific training in handling pathogenic agents and are directed by competent scientists; (2) access to the laboratory is limited when work is being conducted; and (3) certain procedures in which infectious aerosols are created are conducted in biological safety cabinets or other physical containment equipment.

Biosafety Level 3 is applicable to clinical, diagnostic, teaching, research or production facilities in which work is done with indigenous or exotic agents which may cause serious or potentially lethal disease as a result of exposure by the inhalation route. Laboratory personnel have specific training in handling pathogenic and potentially lethal agents and are supervised by competent scientists who are experienced in working with these agents. All procedures involving the manipulation of infectious material are conducted within biological safety cabinets or other physical containment devices or by personnel wearing appropriate personal protective clothing and devices. The laboratory has special engineering and design features.

Biosafety Level 1

Standard Microbiological Practices.

Access to the laboratory is limited or restricted at the discretion of the laboratory director when experiment are in progress.

Work surfaces are decontaminated once a day and after any spill of viable material.

All contaminated liquid or solid wastes are decontaminated before disposal.

Mechanical pipetting devices are used; mouth pipetting is prohibited.

Eating, drinking, smoking, and applying cosmetics are not permitted in the work area. Food may be stored in cabinets or refrigerators designated and used for this purpose only.

Persons wash their hands after they handle materials involving organisms containing recombinant DNA molecules, and animals, and before leaving the laboratory.

All procedures are preformed carefully to minimize the creation of aerosols.

It is recommended that laboratory coats, gowns, or uniforms be worn to prevent contamination or soiling of street clothes.

Special Practices

Contaminated materials that are to be decontaminated at a site away from the laboratory are placed in a durable leakproof container which is closed before being removed from the laboratory.

An insect and rodent control program is in effect.

Containment Equipment

Special containment equipment is generally not required for manipulations of agents assigned to Biosafety Level 1.

Laboratory Facilities

The laboratory is designed so that it can be easily cleaned.

Bench tops are impervious to water resistant to acids, alkalis, organic solvents, and moderate heat.

Laboratory furniture is sturdy. Spaces between benches, cabinets, and equipment are accessible for cleaning..

Each laboratory contains a sink for handwashing.

If the laboratory has windows that open, they are fitted with fly screens.

Biological safety cabinets referred to in this section are classified as Class I, Class II, or Class II cabinets.

A Class I is a ventilated cabinet for personnel protection having an inward flow of air away from the operator.

A Class II cabinet is a ventilated cabinet for personnel and product protection having an open front with inward air flow for personnel protection, and HEPA filtered mass recirculated air flow for product protection.

A Class III cabinet is a closed-front ventilated cabinet of gas tight construction which provides the highest level of personnel protection of all biohazard safety cabinets. The cabinet is fitted with arm-length rubber gloves and is operated under a negative pressure of at least 0.5 inches water gauge.

Release Into the Environment of Certain Plants

Experiments in this category cannot be initiated without submission of relevant information on the proposed experiment to NIH, review by the RAC Plant Working Group, and specific approval by NIH. Such experiments also require the approval of the IBC before initiation. Information on specific experiments which have been approved will be available in ORDA and will be listed in Appendix L-III when the Guidelines are republished.

Approval may be granted by ORDA in consultation with the Plant Working Group without the requirement for full RAC review (IBC review is also necessary) for growing

plants containing recombinant DNA in the field under the following conditions:

The plant species is a cultivated crop of a genus that has no species known to be a noxious weed.

The introduced DNA consists of well-characterized genes containing no sequences harmful to humans, animals, or plants.

The vector consists of DNA: (i) From exempt host-vector systems; (ii) from plants of the same or closely related species; (iii) from nonpathogenic prokaryotes or nonpathogenic lower eukaryotic plants; (iv) from plants pathogens only if sequences resulting in production of disease symptoms have been deleted; or (v) chimeric vectors constructed from sequences defined in (i) to (iv) above. The DNA may be introduced by any suitable method. If sequences resulting in production of disease symptoms are retained for purposes of introducing the DNA into the plant, greenhouse-grown plants must be shown to be free of such sequences before such plants, derivatives, or seed from them can be used in field tests.

Plants are grown in controlled access fields under specified conditions appropriate for the plant under study and the geographical location. Such conditions should include provisions for using good cultural and pest control practices,, for physical isolation from plants of the same species outside of the experimental plot in accordance with pollination characteristics of the species, and for further preventing plants containing recombinant DNA from becoming established in the environment. Review by the IBC should include an appraisal by scientists knowledgeable of the crop, it is production practices, and the local geographical conditions. Procedures for assessing alterations in and the spread of organisms containing recombinant DNA must be developed. The result of the outlined tests must be submitted for review by the IBC. Copies must also be submitted to the Plant Working Group of the RAC.

This summary was prepared by:

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SELECTED REAGENTS AND LABORATORY PROCEDURES

PREPARATION OF AGAROSE GELS

The correct amount of agarose is added to **electrophoresis buffer** and is dissolved by heating in a boiling water bath or a microwave oven. It is important to ensure that all of the agarose has dissolved. This can be accomplished by looking through the solution to ensure that no flakes of agarose are evident. When the agarose has cooled to about 50°C, it is poured into a casting tray.

STAINING OF DNA

Two methods of staining DNA may be used. In one case methylene blue may be used to stain the DNA. The advantage of this method is that it is a much safer method of staining and the disadvantage is that it takes about three to four times as much DNA for bands to show up on the gel. The second method of staining employs the use of ethidium bromide as the staining agent. The advantage of this stain is that it is much more sensitive and it may be use to detect nanogram levels of DNA in the band. The disadvantage is that it is **mutagen** and therefore has to be handled with extreme care.

Staining with Methylene Blue

1% Methylene Blue-- Dissolve 0.5 gm of methylene blue in 50 ml of distilled water.

0.025% methylene blue staining solution -- Add 10 ml of 1% methylene blue to 390 ml of distilled water.

1. Flood the gel with 0.025% methylene blue and allow the gel to stain for 20 - 30 minutes.

2. Rinse the gel in water. Change the water several times. The gel may be destained overnight if a **small volume** of water is used and if the staining tray is covered to prevent evaporation.

3. The stained gel may be viewed over a light box. Covering the light box with plastic wrap will prevent staining the light box.

Staining with Ethidium Bromide

Ethidium bromide is a mutagen and thus must be handled with care. Gloves, goggles and lab coats should be worn when working with this material. It is safer to buy premixed solutions of ethidium bromide to avoid the possibility of breathing the powder. Although there is minimal risk when handling the dilute staining solution, it is recommended that a teacher or qualified scientist handle the ethidium bromide for the student.

When working with ethidium bromide, a restricted sink area should be used. After staining, a funnel should be used to decant the ethidium bromide solution into a storage container so that it may be reused or decontaminated and disposed of properly.

Decontamination of solutions of ethidium bromide using the Quillardet and Hofnung (1988) method

1. Add sufficient water to reduce the concentration of ethidium bromide to less than 0.5 mg/ml.

2. Carefully add 1 volume of 0.5 M $KMnO_4$ and mix carefully. ($KMnO_4$ should be handled in a chemical hood. It is an irritant and is explosive).

3. Add 1 volume of 2.5 N HCl and mix carefully.

4. Allow this solution to stand at room temperature for several hours.

5. Add 1 volume of 2.5 N NaOH and mix carefully. Discard the disabled solution down the drain.

Gels should be disabled in the same manner and discarded.

Stock solution of ethidium bromide -- Add 1 g of ethidium bromide to 100 ml of water. Stir on a magnetic stirrer for several hours to ensure that the dye has dissolved. Wrap the container in aluminum foil and store at 4°C.

Method 1 -- The ethidium bromide is incorporated into both the gel and the running buffer at a rate of 0.5 μg/ml. The gel may be visualized immediately on a ultraviolet transluminator. The disadvantage of this method is that the ethidium bromide cannot be reused as much as in method two and much larger volumes of ethidium bromide solution for decontamination are generated.

Method 2 -- After electrophoresis of the DNA is complete, immerse the gel in electrophoresis buffer containing 0.5 μg/ml ethidium bromide and stain for 45 minutes at room temperature. (The staining solution may be reused several times) The gel is destained in buffer for 5 - 10 minutes.

GEL-LOADING BUFFERS

6X buffer	storage temperature
0.25% bromophenol blue 0.25% xylene cyanol 15% Ficoll (type 400) in distilled water (this buffer is a widely used buffer since it will last for years)	room temperature
0.25% bromophenol blue 0.25% xylene cyanol 40% (W/V) sucrose in water	4°C
0.25% bromophenol blue 0.25% xylene cyanol 30% glycerol in water	4°C
0.25% bromophenol blue 40% (W/V) sucrose in water	4°C

Bromophenol blue and xylene cyanol are used as tracking dyes, since the DNA cannot be seen as it migrates in a gel. Bromophenol blue is the faster moving band an xylene cyanol is the slower moving band.

COMMONLY USED BUFFER FOR AGAROSE GEL ELECTROPHORESIS

Buffer	10X Concentrated Stock		working
TAE (Tris-Acetate)	Tris Base	48.4 gm	0.04 Tris-Acetate
	glacial acetic acid	11.42 ml	0.001 m EDTA
	0.5 M EDTA (pH 8.0)	20 ml	
TBE (Tris-borate)	Tris Base	108 gm	0.089 M Tris-borate
	Boric acid	55 gm	0.089 M Boric Acid
	0.5 M EDTA (pH 8.0)	40 ml	0.002 M EDTA

The pH should be adjusted before adding EDTA. After dissolving the EDTA, bring the stock solution up to volume with distilled H_2O.

TE

10 mM Tris-Cl (pH 8.0)
1 mM EDTA

To prepare TE add Tris base to water, adjust the pH with concentrated HCl, add the EDTA and bring the solution to a proper volume with distilled water.

NESSLERIZATION METHOD OF NITROGEN ESTIMATION

1. EDTA reagent: Dissolve 50 gm EDTA (disodium ethylenediamine tetraacetate dihydrate) in 60 ml water containing 10 gm NaOH. Dilute to 100 ml.

2. Rochelle salt solution: Dissolve 50 gm potassium sodium tartrate tetrahydrate in 100 ml of water. Remove ammonia usually present in the salt by boiling off 30 ml of solution. After cooling dilute to 100 ml.

3. Nessler reagent: Dissolve 100 gm HgI_2 and 70 gm KI in a small quantity of water and add this mixture slowly with stirring, to 160 gm NaOH dissolved in 500 ml water. Dilute to 1 L. Store this solution in the dark in a rubber stoppered borosilicate glass (this solution has a shelf life of up to one year and should yield characteristic color reaction within 10 min).

4. Stock ammonium solution: Dissolve 3.819 gm anhydrous NH_4Cl, dried at 100°C in water, and dilute to 1000 ml. 1.00 ml = 100 mg N = 1.22 mg NH_3.

5. Standard Ammonium solution: Dilute 10.00 ml stock ammonium solution 1000 ml with water. 1ml = 10 μg N = 12.2 μg NH_3.

Sample Analysis

A 50 ml sample or a portion of the sample diluted to 50 ml with water is to be used. If the sample contains sufficient concentrations of calcium, magnesium, or other ions to cause a precipitate with the Nessler reagent or cause turbidity, one drop of either EDTA reagent or one

to two drops of Rochell Salts solution may be added. After mixing well, two ml (if EDTA is used) or one ml (if Rochell salt is used) of Nessler reagent is added.

The samples are mixed by capping the Nessler tubes with clean rubber stopper and inverting at least six times. It is important that temperature and reaction times be the same for the blank (control) samples and standards. The reaction should proceed for 10 minutes unless there is little color development is which case the reaction should proceed for 30 minutes. The color may be measured either photometrically or visually by comparison with standards.

Photometric measurement

The colors that develop may be from yellow to reddish. Yellow Colors represent a low ammonium nitrogen concentration (0.4-5 mg/L) and are measured at a wavelength setting of 400 - 425 nm on the spectrophotometer. Reddish hues are typical of ammonium nitrogen levels of around 10 mg/L and are measured at 450 - 500 nm. A standard curve should be used to determine the concentration in the sample. A new curve should be made for batch of Nessler reagent used.

Standards

Samples for the stand curve should be prepared by adding the following volumes of the standard ammonium chloride solution and diluting the 50 ml with water: 0, 0.2, 0.4, 0.7, 1.0, 1.4, 1.7, 2.0, 2.5, 3.0, 3.5, 4.0, 4.5, 5.0, and 6.0 ml. One ml of Nessler reagent added to each tube and mixed in as previously described.

Calculation

1. Deduct the amount of ammonium -nitrogen in the water used for diluting the original sample before computing the final value.

2. Compute the ammonium -nitrogen by the following equation:

$$\text{mg } NH_3\text{-N/L (51 ml final volume)} = \text{A/ml sample}$$

Abbreviated Nessler Reaction

Oxidize the sample with sulfuric acid and 30% hydrogen peroxide in a Kjeldahl flask calibrated at 50 ml. Dilute to volume and take an aliquot representing 20-50% of the sample. Add 2 ml of Nessler solution and dilute to 50 ml. Incubate for 15 minutes, read a 450 nm, and deduct the value of the blank.

ANTIBIOTIC SOLUTIONS

ANTIBIOTIC	STOCK SOLUTIONS CONCENTRATION	STORAGE
Ampicillin: sodium salt	50 mg/ml in H_2O	-20°C
Chloramphenicol	34 mg/ml in ethanol	-20°C
Kanamycin	10 mg/ml in H_2O	-20°C
Streptomycin sulfate	10 mg/ml in H_2O	-20°C
Tetracycline*	12.5 mg/ml tetracycline hydrochloride in ethanol/water (50% V/V)	-20°C
neomycin	5 mg/ml	-20°C

ANTIBIOTIC	WORKING CONCENTRATIONS STRINGENT[1] PLASMIDS	RELAXED[2] PLASMIDS	UNSURE OF PLASMID TYPE
ampicillin: sodium salt	20 μg/ml	60 μg/ml	35-50 μg/ml
chloramphenicol	25 μg/ml	170 μg/m	130 μg/ml
kanamycin	10 μg/m	150 μg/m	125-50 μg/ml
streptomycin sulfate	10 μg/m	150 μg/m	125 μg/ml
tetracycline*	10 μg/m	150 μg/ml	12.5-15.0 μg/ml
neomycin			5 μg/ml

*Note. Because tetracycline is light sensitive, solutions and plates containing this antibiotic should be stored in the dark. Usually one wraps the tubes in plates in aluminum.

*Note. Magnesium ions are antagonists of tetracycline. Media without magnesium salts (eg. LB) should be used to select for bacteria that are resistant to this antibiotic.

It is best (but not necessary) to filter sterilize the stock solutions.

1. There are usually one to five copies of these plasmids per cell.
2. There are thirty to fifty copies per cell.

ADDITION OF ANTIBIOTICS TO MEDIA

Antibiotics should not be added to media before sterilization. They should be added after the sterilized media has been allowed to cool to 55 - 60°C. (If the solution cools too long in the case of an agar media, the agar will begin to solidify and it will be necessary to reheat it and again allow it to cool before adding the antibiotic).

LB Medium

bacto tryptone	10 gm
NaCl	10 gm
Bacto yeast extract	5 gm
distilled water	1 liter volume

adjust pH to 7.0 with 5M NaOH

LB AGAR

Bacto agar	15 gms/liter of LB Medium

BG-11 Medium

Label Stock	Stock	For 1 L complete	MW
#1 $NaNo_3$	150.0 g/l	10 ml	84.99
#2 $CaCl_2$-2 H_2O	36.0 g/l	1 ml	147.0
#3 2x $FeNH_4$ citrate	12.0 g/l	1 ml	299.05
#4 EDTA-Na_2	1.0 g/l	1 ml	372.3
#5 K_2HPO_4	40.0 g/l	1 ml	174.2
#6 $MgSO_4$-7 H_2O	75.0 g/l	1 ml	246.5
#7 Na_2CO_3	20.0 g/l	1 ml	105.9
#8 micronutrients	see recipe	1 ml	
H_2O	to 1 liter		

Add given amounts of stock solutions (stored in refrigerator) to container with some water. Finally bring to volume and autoclave.

BG-11 Micronutrients

	MW	Stock	Conc
H_3BO_3	61.83	2.86 g/l	46.3 μM
$MnCl_2$-4 H_2O	179.9	1.81 g/l	9.1
$ZnSo_4$-7 H_2O	287.5	0.222 g/l	0.77
Na_2MoO_4-2 H_2O	241.9	0.39 g/l	1.61
$CuSO_4$-5 H_2O	249.7	0.079 g/l	0.32
$Co(NO_3)_2$-6 H_2O	291.0	0.0494 g/l	0.17

Dissolve each salt before adding the next one. The solution should be perfectly clear.

Slants/top agar: 7.5 g agar/ 500 ml; autoclave 25-30 minutes (250 ml= 42 tubes @ 5 ml /tube)

Plates (this recipe gives plates stiff enough to add antibiotic under at some time following transformation; alternatively less agar (1X - 11 g/l) may be used if antibiotic is added in top agar):

Autoclave two flasks separately and then mix them together and cool to approximately 60°C, prior to pouring plates each of which will have approximately 40 ml. Flask #1 contains 2X agar (34 g/l; 1X - 17 g/l) (use a flask with room enough to add the contents of flask #2 after autoclaving, heat to melt agar and mix before autoclaving); Flask #2 contains 2X BG-11. You may include a stir bar in one solution to facilitate mixing sterile solutions. The efficiency of transformation is increased by adding 10 ml/l of a 100mM stock of filter sterilized sodium thiosulfate (less than one week old) to the BG-11 agar <u>after</u> autoclaving but prior to pouring plates.

-- Prepared by Dr. Carolynn Vann
and Jo Ann Meunier

Basic Rules for Handling Enzymes

[**Note:** This is an update of our popular primer which was first published in the December 1985 issue of BMBiochemica.]

For the novice—basic hints to guide you through your first enzymatic reaction.

For the expert—a refresher course and an aid for training your students.

1. For best stability, enzymes should be stored in their original commercial form (lyophilized, ammonium sulfate suspension, etc.), undiluted and at the appropriate temperature as specified on the label.
2. For enzyme solutions and assay buffers, use the highest purity H_2O available. Glass distilled H_2O is best. Deionized H_2O, especially if passed through an old deionizing filter or a reverse osmosis device, may contain traces of organic contaminants which inhibit enzymes.
3. Enzymes should be handled in the cold (0 to +4°C). Dilute for use with ice-cold buffer or distilled water, as appropriate for each enzyme. While using the enzyme solution or suspension at the bench, keep it in an ice bath or ice bucket.
4. Dilute enzyme solutions are generally unstable. The amount of enzyme required for the experiment should be diluted within 1–2 hours of use. Enzymes should not be diluted for long-term storage.

   Enzymes, especially those which have been diluted, should be checked for activity periodically to ensure that any slight loss in activity is taken into account when designing an experimental protocol. Our products are guaranteed through the control date printed on the vial only when stored in the original form and at the correct temperature.
5. Do not shake crystalline suspensions (*e.g.*, ammonium sulfate suspension) since oxygen tends to denature the enzyme. The material should be resuspended with gentle swirling or by rolling the bottle on the laboratory bench. Once the enzyme crystals have been uniformly resuspended, remove the amount needed with a pipette. In many cases, the enzyme crystals may be used directly in assay procedures.
6. Do not freeze crystalline suspensions. Freezing and thawing in the presence of high salt causes denaturation and loss of activity.
7. Vials containing lyophilized enzymes (as well as cofactors, such as NADH and NADPH) should be warmed to room temperature before opening. This prevents condensation of moisture onto the powder, which can cause loss of activity or degradation. If the reagent is hygroscopic, one such mishandling may well ruin the entire vial.
8. Avoid repeated freeze-thawing of dilute enzymes and lyophilizates in solution. Store in small aliquots. Thaw one portion at a time and store that portion, once thawed, at +4°C. The stability of individual enzymes may vary greatly and often should be determined empirically under your exact conditions.
9. Detergents and preservatives should be used with caution, since they may affect enzyme activity. Sodium azide, for example, inhibits many enzymes which contain heme groups (*e.g.*, peroxidase). Detergents added at concentrations above their critical micellar concentration form micelles which may entrap and denature the enzyme.
10. Enzymes should be handled carefully to avoid contamination of any kind. Use a fresh pipette for each aliquot that is removed from the parent vial. Never return unused material to the parent vial. Wear gloves to prevent contaminating the enzyme with proteases, DNases, RNases and inhibitors often found on fingertips. Never pipette by mouth.
11. Adjust the pH of the enzyme buffer at the temperature at which it will be used. Many common buffers (Tris, glycylglycine, Bes, Aces, Tes, Bicine, Hepes) change pH rapidly as the temperature changes. For instance, Tris buffer decreases 0.3 pH units for every 10°C rise in temperature. A solution of Tris, adjusted to pH 7.5 at +25°C, will have a pH of 8.1 at +4°C or 7.2 at +37°C. The change in pH per 10°C temperature change for other buffers is: Aces, −0.20; Bes, −0.16; Bicine, −0.18; glycylglycine, −0.28; Hepes, −0.14; Tes, −0.20 [Good, N.E. *et al.* (1966) *Biochemistry* 5:467].
12. The absorbance at 280 nm, widely used to quickly determine the protein concentration of an enzyme solution, actually is due to the presence of tyrosine and tryptophan in the protein. If an enzyme (*e.g.*, superoxide dismutase) contains a low amount of these two amino acids, it will not absorb significantly at 280 nm.

Detailed information is available on many enzymes. The most complete references are:

Methods in Enzymology, published by Academic Press, Editors-in-chief: Sidney P. Colowick and Nathan O. Kaplan. There are more than 165 volumes in this series, covering an extensive range of topics.

The Enzymes, 3rd edition, edited by Paul D. Boyer. An excellent, broad series which focuses more on the physical and biological properties of the enzyme and less on methodology than *Methods in Enzymology*.

Methods of Enzymatic Analysis, 3rd edition, Editor-in-chief: Hans U. Bergmeyer, published by Verlag Chemie. In-depth discussions of techniques of analysis which use enzymes or which assay enzymatic activity.

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GLOSSARY

acid. (1) A substance that increases the hydronium ion concentration of its aqueous solution (2) a proton donor (3) an electron-pair acceptor.

acrylamide. Monomer of the form $CH_2CH\ CONH_2$ to make poly-acrylamide gel.

acyl group. A radicle having the general formula RCO-, derived from an organic acid.

acylation. The addition of acyl group.

adventitious. Developing from unusual points of origin, such as shoots or root tissues from callus or embryos from sources other than zygotes. This term can also be used to describe agents which contaminate cell cultures.

aerobic. Utilizes oxygen in respiration.

agar. An extract from red algae that is used to produce a semi-solid microbiological media.

agarose. A highly purified colloidal extract from red algae, used to make gels.

agarose gel electrophoresis. A matrix composed of a highly purified form of agar is used to separate larger DNA and RNA molecules.

agronomy. The application of scientific principles to the cultivation of crops.

alcohol. A compound containing a hydrocarbon group and one or more hydroxyl group, (-OH).

alginate. Alginate is a salt of alginic acid which is a hyrdrophilic colloidal polysaccharide found in several species of brown algae.

alpha amylase. A starch digesting enzyme.

amino acid. Any organic molecule of the general formula $R\text{-}CH(NH_2)COOH$, having both acidic and basic properties. Amino acid are the building blocks of proteins.

ampholyte. A molecule that contains at least one acidic and one basic group.

amphoteric substance. A molecule that acts like ampholyte.

ampicillin (ß-lactamase). An antibiotic derived from penicillin that prevents bacterial growth by interfering with cell wall synthesis.

amplify. To increase the number of copies of a DNA sequence, *in vivo* by inserting into a cloning vector that replicates within a host cell or *in vitro* by polymerase chain reaction (PCR).

anaerobic. Living or functioning in the absence of oxygen.

antibody. An immunoglobulin protein produced by ß-lymphocytes of the immune system that binds to a specific antigen molecule.

antibiotic. A class of natural and synthetic compounds that inhibit the growth of or kill microorganisms.

antibiotic resistance. The ability of a microorganism to produce a protein that disables an antibiotic or prevents transport of the antibiotic into the cell.

artifical seed. A somatic embryo within a ocoating. Artificial seeds can be planted in an analogous manner to a seed and are similar to true seeds, with the exception that they are clonal propagules.

asepsis. Without infection or contaminating microorganisms.

aseptic technique. Procedures used to prevent the introduction of fungi, bacteria, viruses, mycoplasma or other microorganisms into cell, tissue and organ cultures. Although these procedures are used to prevent microbial contamination of cultures, they also prevent cross contamination of cell cultures as well. These procedures may or may not exclude the introduction of infectious molecules.

assay. A procedure for measurement or identification.

auxotroph. A mutant microorganism that will grow in a minimal media only when it is supplemented with a growth factor or factors (such as amino acids) not required by the normal parent.

bacteriocin. A toxin or antibiotic, produced by one class of bacteria which kills other, usually closely related, bacteria.

bacterium. A single-cell prokaryotic organism.

bacteriophage (phage or phage particle). A virus that infects bacteria. Altered forms are used as vectors for cloning DNA.

base. A molecule that can accept a hydrogen ion and become positively charged; a molecule which releases OH. In biochemistry of ten refers to the nitrogenous bases (eg. adenine and thymine) of nucleotides.

base pair (bp). A pair of complementary nitrogenous basis in a DNA molecule (adenine-thymine and guanine-cytosine). Also, the unit of measurement for DNA sequences.

biodegradation. The breaking down of organic compound by microbes.

biomass. Total weight of organisms per unit area or per culture.

biopolymer. A polymer produced by a living organism.

bioprocess. A process in which living cells or components thereof, are used to produce a desired end product.

bioreactor. A vessel used for bioprocessing.

Biotechnology. Any technique that uses living organisms or parts of organisms to make or modify products, to improve plants or animals, or to develop microorganisms for specific uses.

biotin-label. The attachment of the vitamin biotin to DNA so that DNA might be used to detect the presence of a specific sequence of DNA.

blot. As a verb it means to transfer DNA, RNA, or protein to an immobilizing matrix such as nitrocellulose membrane.

bovine. Belonging to or derived from the genus *Bos* of ruminant mammals (cows).

bromophenal blue. Water soluble tracking dye.

buffer. A solution which minimizes changes in hydrogen ion concentration which would otherwise occur as a result of chemical reaction.

callus. A mass of undifferentiated plant cells manipulated in plant tissue culture.

carcinogen. A substance that induces cancer.

carotenoid. A class of easily oxidizable yellow, orange, red or pigments that are widely distributed in plants and animals and are soluble in fats and fat solvents.

catabolism. The breakdown of complex organic molecules by living organisms with release of energy.

catabolite. Any product of catabolism.

cation. A positively charged ion.

catalyst. A substance that promotes a chemical reaction by lowering the activation energy of a chemical reaction, but itself remains unaltered at the end of the reaction.

cell culture. Term used to denote the maintenance or cultivation of cells in vitro including the culture of single cells. In cell cultures, the cells are no longer organized into tissues.

cellobiase. An enzyme which breaks down cellobiose.

cellobiose ($C_{12}H_{22}O_{11}$). A disaccharide which does not occur freely in nature.

cellulase. An enzyme that digests cellulose.

centrifugation. Separating molecules by size or density using centrifugal forces generated by a spinning rotor.

cDNA library. A library composed of complementary copies of cellular mRNAs.

chelating agent. An organic compound which bonds with metals in solution.

chitin. A polysaccharide found in the shells of invertebrates such as lobsters and in the exoskeleton of insects.

chitinase. An enzyme that breaks down chitin.

chitosan. Partially deacylated chitin that makes a good substrate for chitinase.

chloramphenicol. An antibiotic that interferes with protein synthesis.

chromosome. (1) in eukaryotes, an independent nuclear body carrying genetic information in a specific linear order and consisting of DNA and associated proteins (2) in prokaryotes an analogous circular DNA molecule (3) the analogous DNA or genetic RNA of virus.

chromotagraphy. A technique for analyzing or separating mixtures of gases, liquids, or dissolved substances. This technique utilizes the dissolved substances passing (moving phase) over an absorbent material (stationary phase).

clone. This term is used in a number of senses. As a noun it may mean (1) a population of recombinant DNA molecules all carrying the same inserted sequence, or (2) a population of cells or organisms of identical genotype. It is most frequently used to describe a colony of microorganisms which harbour a specific DNA fragment inserted into a vector molecule. As a verb 'to clone' means to use *in vitro* recombination techniques to insert a particular gene or other DNA sequence into a vector molecule. In animal cell culture terminology, a population of cells derived from a single cell by mitoses. A clone is not necessarily homogeneous and, therefore, the terms clone and cloned do not indicate homogeneity in a cell population, genetic or otherwise. In plant culture terminology, the term may refer to a culture derived as above or it may refer to a group of plants propagated only by vegetative and asexual means, all members of which have been derived by repeated propagation from a single individual.

codon. A group of three nucleotides that specifies addition of one of the 20 amino acids during translation of an mRNA into a polypeptide.

colony. A uniform mass of cells derived from a single cell growing on a solid surface.

colorimetry. Any technique by which an unknown color is evaluated in terms of standard colors.

competency. An ephemeral state, induced by treatment with cold cations, during which bacterial cells are capable of uptaking foreign DNA.

concanavalin A. An anti-body-like protein found in jack beans (*Canavalia ensiformis*).

conjugation. The act of joining together; in bacteria a process in which genetic information is transferred from one cell to another by direct cell contact.

copy number. The number of replicates of a particular plasmid found in a cell.

cosmid. A plasmid vector containing a COS site that enables it to be packaged into infective lambda bacteriophage particles. Used for cloning DNA sequences of 35,000-45,000 bp.

cotyledon. A food-storing structure in dicot seeds, sometimes emerging as first leaves; food-digesting organ in most monocot seeds; first leaves in gymnosperm leaves.

cultivar. A horticulturally or agriculturally derived variety of a plant as distinguished from a natural variety.

cyanobacteria. Formerly blue-green algae. Any of a large group of blue--green photosynthetic prokaryotes having as photopigments chlorophyll a, phycocyanin, and phycoerythrin; and producing oxygen as a photosynthetic waste product.

cyanophage. A bacteriophage which has a cyanobacterium as its host.

cytokinin. A class of plant growth hormones important in the regulation of nucleic acid and protein metabolism, in cell division, delaying senescence, and organ initiation.

dalton. A unit of atomic and molecular mass, equivalent to one twelfth the mass of an atom of carbon 12.

DEAE-cellulose (Diethylaminoethyl cellulose). A positively charged resin used in ion exchange chromatography; an anion exchanger.

denature. To induce structural alternations that disrupt the biological activity of a molecule. Often refers to breaking hydrogen bonds between base pairs in double-stranded nucleic acid molecules to produce in single-stranded polynucleotides or altering the secondary and tertiary structure of a protein, destroying its activity.

dextrans. Polysaccharides composed of branched chains of glucose which serve as storage products in bacteria and yeasts.

digest. To cut DNA molecules with one or more restriction endonucleases.

dimer. Double molecules consisting of two identical subunits or monomers.

dimerization. The formation of a dimer.

DNA (deoxyribonucleic acid). An organic acid polymer composed of four nitrogenous bases -adenine, thymine, cytosine, and guanine- linked via intervening units of phosphate and the pentose sugar deoxyribose. DNA is the genetic material of most organisms and usually exists as a double-stranded molecule.

DNA polymerase. Synthesizes a double-stranded DNA molecule using a primer and DNA as a template.

DNA probe. A short section of a single-stranded DNA designed to be complementary to some characteristic part of the DNA to be tested. Probes are labeled, with radioactivity for example, so that their binding can be easily detected. Such probes have great potential for fast, accurate diagnosis of disease.

DNase. A class of enzymes that degrades DNA.

dodecyl ($C_{12}H_{25}$). Derived from dodecane by removing one hydrogen atom.

dominant gene. A gene which fully expresses its phenotype.

double helix. Describes the coiling of the antiparallel strands of the DNA molecule, resembling a spiral staircase in which the paired bases form the steps and the sugar-phosphate backbones form the rails.

EDTA (ethylenediamine tetraacetate). A chelating agent, used in biochemical systems *in vitro* for the chelating of divalent metal ions.

electrophoresis. The technique of separating charged molecules in a matrix to which is applied an electrical field.

eluant. A liquid used to extract one material from another.

elution. The removal of adsorbed molecules from a porous bed or chromatographic column by means of a stream of liquid or gas.

embryo. A young organism in the first stages of development.

embryogenesis. The formation and development of an embryo from an egg.

endoglucanase. Enzymes which breaks bonds between the subunits within a glucan chain.

endonuclease. An enzyme which cuts within a polynucleotide chain.

enzyme. Any group of catalytic proteins that are produced by living cells and that mediate and promote chemical processes of life without themselves being altered or destroyed.

epitope. Major antigenic site on an antigen that relates specifically to immunity.

eppendorf. The name of a West German instrument manufacturer which has entered the language of molecular biologists. The name is used to describe the small tubes used in a bench-top centrifuge and to describe disposable plastic tips used with micropipets. As with other such names such as "kleenex", the name is used irrespective of who actually manufactured it.

episome. Autonomous self replicating DNA in bacterial which can integrate into the bacterial chromosome semipermanently.

ergosterol. Provitamin D_2, it is the main sterol of yeast. It is converted by irradiation with UV to vitamin D_2.

escherichia coli (E. coli). A bacterium from the gut of humans which has been the most thoroughly researched of all bacterial cells.

esterases. Any hydrolytic enzymes that attack an ester, splitting off the acid.

esters. Compounds formed by acids and alcohols (eg. fats and oils).

ethidium bromide. A fluorescent dye used to stain DNA and RNA. The ethidium bromide molecule intercalates (stacks) between nucleotides, and the ethidium bromide-nucleic acid complex fluoresces when exposed to ultraviolet light.

eukaryote. An organism whose cells possess a nucleus and other membrane-bound vesicles, including all members of the protist, fungi, plant, and animal kingdoms.

explant. Tissue taken from its original site and transferred to an artificial medium for growth or maintenance.

explant culture. The maintenance or growth of an explant in culture.

extrachromosomal. DNA molecules such as plasmids in bacteria which are independent of the chromosomes.

fermentation. An enzymatic transformation of organic substances, especially carbohydrates. It is often accompanied by the production of gas.

fibroblast. A precursor cell of connective tissue that is relatively easy to maintain in cell culture.

ficoll. A copolymer of sucrose and epichlorohydrin. It has a molecular weight of approximately 400,00 and it used in loading buffers.

flocculating agent. A chemical which will cause the aggregation of material into large clumps.

fluorometer. An instrument that measures the fluorescent radiation emitted by a sample which is exposed to monochromatic radiation.

ß-galactosidase. An enzyme which will cleave lactose into glucose and galactose.

gel. The inert matrix used for electrophoretic separation of nucleic acids or protein.

gene. A locus on a chromosome that encodes a specific protein or several related proteins.

gene expression. The process of producing a protein from its DNA-and mRNA-coding sequences.

genetic engineering. A term for the use of *in vitro* techniques to produce DNA molecules containing novel combinations of genes or other sequences.

genome. The full haploid complement of genetic information of a diploid organism; the full complement of genetic information of a prokaryote.

glucans. Polysaccharides composed of D-glucose.

glucose oxidase. An enzyme swhich oxidizes ß-D-glucose in the presence of oxygen to glucuronic acid and hydrogen peroxide.

glucuronic acid ($C_6H_{10}O_7$). Acid resulting from oxidation of the CH_2OH radical of D-glucose to COOH; A component of many polysaccharides in plant cell walls, bacterial capsules.

glucuronidase. An enzyme that catalyzes hydrolysis at glucuronides.

glucuronide. A compound resulting from the interaction of glucuronic acid with a phenol, an alcohol, or an acid containing a carboxyl group.

haploid. Cells having one set of chromosomes.

hemicellulases. Enzymes which break down hemicelluloses.

hemicellulose. A type of polysaccharide found in plant cell walls in association with cellulose and lignin; it is soluble in and extractable by dilute alkaline solutions.

herbicide. Any substance that is toxic to plants; usually used to kill specific unwanted plants.

host. An organism able to support the replication of a plasmid or virus.

hybrid. The offspring of two parents differing in at least one genetic characteristic (trait). Also, heteroduplex DNA or DNA-RNA molecule.

hybridization. The hydrogen bonding of complementary DNA and/or RNA sequences to form a duplex molecule.

hydrogen bond. A relatively weak bond formed between a hydrogen atom (which is covalently bound to a nitrogen or oxygen atom) and a nitrogen or oxygen with an unshared electron pair.

hydrolysis. A reaction in which a molecule of water is added at the site of cleavage of a molecule to two products.

hydronium ion. A hydrated proton; the H_30^+ ion.

inducer. A chemical or physical agent which, when given to a population of cells, will increase the amount of transcription from specific genes (eg. isopropythio-ß-galactoside is a powerful inducer of the lac operon).

inducible. A gene or gene-product is said to be inducible it its transcription of synthesis is increased by exposure to the cells to an effector. These are usually small molecules whose effects are specific to particular operons or groups of genes.

initiation codon, initiator. The codon which specifies the first amino acid of a polypeptide chain. In bacteria, the initiation codon is either AUG, which is translated a n-formyl methionine (a modified amino acid) or, rarely, GUG (valine). In eukaryotes the initiation codon is always AUG and is translated as methionine. The term is also used to describe the corresponding sequence in DNA, ATG.

inoculum. A small amount of substance containing a microbe from a pure culture which is used to start a new culture.

insert. The piece of foreign DNA introduced into a vector molecule.

insertion site, cloning site. A unique restriction site in a vector DNA molecule into which foreign DNA can be inserted. The term is also used to describe the position of integration of a transposon.

insertional inactivation. The technique in which foreign DNA is cloned into a restriction site which lies within the coding sequence of a gene in the vector. The insertion of foreign DNA at such a site interrupts the gene's sequence such that its original function is no longer expressed. This permits the detection of recombinant molecules following transformation.

in vitro. Literally "in glass," refers to the recreation of biological processes in an artificial environment.

in vivo. Refers to biological processes that take place within a living organism or cell.

IPTG (Isopropyl-ß-D-thiogalactopyranoside). This chemical is used as an inducer of the lac operon for ß-galactosidase. It has a formula of $C_9H_{18}O_5S$ and a molecular weight of 238.3.

ion. A charged atom or molecule.

ion-exchange chromatography. A chromatographic procedure in which the stationary phase consists of ion-exchange resins which may be acidic or basic. Hydrated ions of a solid phase are exchanged for ions of a like charge in solution.

isoelectric focusing. A method for the fractionation of amphoteric substances according to their isoelectric points. When a pH gradient is created, amphoteric substances will focus at that region in the pH gradient that corresponds to their isoelectric point.

isoelectric point. A pH at which any amphoteric molecule such as an ampholyte, protein or polypeptide has a zero net electrical charge.

isoenzyme. Any electrophoretically distinct form of an enzyme, representing variation in the enzyme structure but having the same substrate specificity.

kanamycin. An antibiotic of the aminoglycoside family that poisons translation by binding to the ribosomes.

karyotype. The total chromosme constitution of an individual.

K12. A laboratory strain E. coli used in many experiments.

lac. An abbreviation for lactose.

linalool (3,7-Dimethyl-1,6-Octadien-3-ol). This terpene is a colorless liquid. It boils at 195-196°C has a molecular weight of 154.24 and its formula is $C_{10}H_{18}O$. It is the chief constituent of linaloe oil. It also occurs in the oil of other plants.

Luekemia. A liquid tumor characterized by the overproduction of nonfunctional or immature white blood cells.

library. A collection of cells, usually bacteria or yeasts, that have been transformed with recombinant vectors carrying DNA inserts from a single species.

ligase (DNA ligase). An enzyme that catalyzes a condensation reaction that links two DNA molecules via the formation of a phosphodiester bond between the 3' hydroxyl and 5' phosphate of adjacent nucleotides.

ligate. The process of joining two or more DNA fragments.

lignin. A complex organic material that strengthens plant cell walls.

lignification. Hardening of plant tissue from wall deposition of lignin.

lipid. A molecule generally composed of glycerol linked to fatty acids by means of ester bonds.

lysis. Rupture or dissolution of a cell.

lysozyme. An enzyme that lyses bacteria by

breaking down the cell wall. In particular this enzyme attacks the peptidoglycan layer of the wall.

mapping. Determining the physical location of a gene or genetic marker on a chromosome.

marker. A mutation in a gene which facilitates the study of its inheritance.

megadalton (Md). 10^6 daltons of molecular weight.

metabolism. The biochemical processes that sustain a living cell or organism.

microbe. A microorganism.

microbiology. The science dealing with the study of microorganisms.

microgram (μg). 10^{-6} grams.

microliter (μl). 10^{-6} liters.

micromole (μM). 10^{-6} moles.

microorganisms. Any microscopic organisms including bacteria, viruses, unicellular algae, protozoans, and microscopic fungi.

mitogen. An agent that causes cells to divide and multiply; A stimulant to mitosis.

mRNA (messenger RNA). Directly transcribed from nuclear DNA then translated into polypeptides at the ribosomes.

mole. The quantity of a chemical substance that has a mass in grams numerically equal to its molecular mass in daltons; 6.023×10^{23} molecules of a substance.

molecular biology. The study of the biochemical and molecular interactions within living cells.

molecular genetics. The study of how genes function to control cellular activities.

monoclonal antibodies. Immunoglobulin molecules of single-epitope specificity that are secreted by a clone of ß cells.

monocotyledon (monocot). A flowering plant whose embryo possesses one cotyledon, or seed leaf, including all the staple cereal crops, grasses, lilies, and palms.

mutagen. An agent which causes mutations.

mutant. A phenotypic variant resulting from a changed or new gene.

mutation. An alteration in DNA structure or sequence of a gene.

neomycin. An antibiotic produced by *streptomyces fradine*. It is active against gram positive bacteria and some gram negative bacteria.

nitrocellulose. A membrane used to immobilize DNA, RNA, or protein, which can then be probed with a labeled sequence or antibody.

nitrogenous bases. The purines (adenine and guanine) and pyrimidines (thymine, cytosine, and uracil) that comprise DNA and RNA molecules.

Northern hybridization (Northern blotting). A procedure in which RNA fragments are transferred from an agarose gel to nitrocellulose filter, where the RNA is then hybridized to a probe.

nucleotide. A building block of DNA and RNA, consisting of a nitrogenous base, a five-carbon sugar, and a phosphate group.

nucleus. The membrane-bound region of a eukaryotic cell that contains the chromosomes.

oligonucleotide. A DNA polymer composed of only a few nucleotides.

oncogene. A gene that contributes to cancer formation when mutated or inappropriately expressed.

ONPG (2-Nitrophenyl-ß-D-Galactopyranoside). This compound is a substrate for the determination of ß-galactosidase. It has a formula of $C_{12}H_{15}NO_8$ and a molecular weight of 301.3.

operator. A gene that is adjacent to a structural gene or to a group of contiguous structural genes and that controls the transcription of the gene or group of genes; The operator interacts with a specific repressor protein, thereby controlling the functioning of the gene or genes.

operon. A region of DNA that includes structural genes and the genes controlling them.

origin of replication. The nucleotide sequence at which DNA synthesis is initiated.

passage. The transfer or transplantation of cells, with or without dilution, from one culture vessel to another. It is understood that any time cells are transferred from one vessel to another, a certain portion of the cells may be lost and, therefore, dilution of cells, whether deliberate or not, may occur. This term is synonymous with the term "subculture".

pectin. A group of highly variable complex polysaccharides found in middle lamella of plant primary cell walls, rich in galacturonic acid, and forming a gel when isolated, and also containing arabinose, galactose and rhamnose residues.

pectinase. Commercially usually a mixture of glycosidases that hydrolyse the various components of pectin; sometimes refers to polygalacturonase.

peptide bond. The bond that forms between a carboxyl group (COOH) one of amino acid and an amino group of another amino acid.

peptone. Polypeptides; the product of hydrolysis of proteins by enzymes such as pepsin.

peroxidase. Enzymes which act on hydrogen peroxide in the delydrogenation of various substrates. The hydrogen peroxide is reduced to water.

pH. Strictly defined, it is the negative logarithm of hydrogen ion concentration in a water based solution. A term which refers to the concentration of hydrogen ions in a solution.

phenotype. The observable characteristics of an organism produced by the interaction of genotype and the environment.

plant tissue culture. The growth or maintenance of plant cells, tissues, organs or whole plants *in vitro*.

polyacrylamide gel electrophoresis. Electrophoresis through a matrix composed of a synthetic polymer, used to separate proteins, small DNA, or RNA molecules of up to 1000 nucleotides.

polyethylene glycol, (PEG). This polymer is available in a range of molecular weight from ca. 1000 to ca. 6000. Peg 4000 + 6000 are commonly used to promote cell or protoplast fusion and to facilitate DNA uptake in transformations of yeast. The polymer has the general formula: $HOCH_2(CH_2OCH_2)_xCH_2OH$.

polymer. A generally large molecule formed by the union of simple molecules (monomers).

polymerase. An enzyme that catalyzes the addition of multiple subunits to a substrate molecule.

polysaccharide. A polymer composed of multiple units of monosaccharide (simple sugar).

primer. A short DNA or RNA fragment annealed to single-stranded DNA, from which DNA polymerase extends a new DNA strand to produce a duplex molecule.

promoter. The site on the DNA molecule to which RNA polymerase attaches and at which transcription is initiated.

protein. A polymer of amino acids linked via peptide bonds and which may be composed of two or more polypeptide chains.

proto-oncogene. A normal cellular gene capable of conversion to an oncogne (capable of causing cancer).

proteolytic. The ability to break down protein molecules.

protoplast. A cell from which the entire cell wall has been removed. This term is used to describe such plant, bacterial or fungal cells.

pure culture. *In vitro* growth of only one type of microorganism.

Recombinant DNA (rDNA). The hybrid DNA formed by combining pieces of DNA from different types of organisms.

recessive gene. Characterized as having a phenotype expressed only when both copies of the gene are mutated or missing.

reducing sugar. Sugars that possess the property of readily reducing akaline solutions of many metallic salts such as copper, silver or bismuth; (eg. monosaccharides and most disaccharides including lactose).

relaxed replicating plasmids. These plasmids require the activity of DNA polymerase I and can replicate in absence of protein synthesis. There are usually 30 - 50 copies of these plasmids per cell.

repressor. A DNA-binding protein in prokaryotes that blocks gene transcription by binding to the operator.

restriction endonuclease (enzyme). A class of endonucleases that cleaves DNA after recognizing a specific sequence.

restriction fragment. The individual polynucleotides produced by the digestion with a restriction endonuclease.

restriction -fragment-length polymorphism (RFLP). Differences in nucleotide sequence between alleles at a chromosomal locus result in restriction fragments of varying lengths detected by Southern analysis.

restriction map. A physical map showing the locations of restriction enzyme recognition sites.

reverse transcriptase. An enzyme capable of directing the production of a singe strand DNA copy from an RNA template.

seed. The fertilized and ripened ovule of a plant; comprised of an embryo, cotyledons (one or two) as food supply and a surrounding hard seed coat.

selectable marker. A gene whose expression allows one to identify cells that have been transformed or transfected with a vector containing the marker gene.

selective medium. Nutrient material constituted such that it will support the growth of specific organisms while inhibiting the growth of others.

sepharose. A bead-formed gel prepared from agarose. The agarose used in sepharose has been purified in order to remove charged polysaccharides found in agarose, thus removing most charged groups from the agarose. Sepharose is used to separate molecules based on molecular size.

serial dilution. A technique for measuring the number of suspended microbes in a fluid by progressively diluting the fluid until the density of microbes is small enough for accurate counts to be made.

shuttle vector. A vector molecule which is able to replicate in two different host organisms and can therefore be used to "shuttle" genes from one host to another.

sodium lauryl sulfate ($CH_3(CH_2)CH_2OSO_3NA$). A water-soluble salt which is used as a detergent.

somatic embryogenesis. Production of plant strutures (somatic embryos) consisting of a radicle, shool, and cotyledons all with vascular connections. Somatic embryos are entical to zygotic (seed) embryos in terms of morphology, biochemistry, dessication ability, and ability to germinate and form complete plants. Unlike zygotic embryos, somatic embryos are not derived as a result of meiosis but from somatic tissue as a result of mitosis.

Southern hybridization (Southern blotting). A procedure in which DNA restriction fragments are transferred from an agarose gel to a nitrocellulose filter, where the denatured DNA is then hybridized to a probe.

species. A taxonomic group composed of individuals having closely related characteristics; in sexually reproducing organisms, a group whose members are at least potentially able to breed with one another but are unable to breed with members of any other group.

strain. A pure culture of organisms within a species, characterized by one or more particular physical or genetic properties.

stringent replicating plasmids. These plasmids require protein synthesis and the activity of DNA polymerase III. There are usually one to five copies of these plasmids per cell.

supernatant. The soluble liquid fraction of a sample after centrifugation or precipitation of insoluble solids.

TEMED (N,N,N'N'-Tetramethylethylenediamine). A catalyst for the polymerization of acrylamide gels.

terpene. A class of natural unsaturated hydrocarbons with formulae $(C_5H_8)_n$, found in plants.

tetracycline. An antibiotic that interferes with protein synthesis in prokaryotes.

thermophiles. Microorganisms growing best at temperatures between 45 and 70°C.

tissue culture. Placement of tissues, cells, or organs into a sterile laboratory (in vitro) environment. Tissue culture is done to produce specific compounds from the cells, to study metabolism, to transform cells, and to regenerate plants, among others.

transcription. The process by which the genetic information contained in DNA is transferred to a molecule of messenger RNA. This is the first step in protein synthesis.

transduction. The transfer of bacterial genetic information from one cell to another by a bacteriophage.

transformation. In prokaryotes, the natural or induced uptake and expression of foreign DAN sequence--typically a recombinant plasmid in experimental systems. In higher eukaryotes, the conversion of cultured cells to a malignant phenotype--typically through infection by a tumor virus or transfection with an oncogene.

transgenic organism. An organism formed by the insertion of foreign genetic material into the germ line cells of organisms.

translation. The process by which genetic information encoded in messenger RNA (mRNA) in the form of a sequence of base triplets (codons) is converted into a sequence of amino acids in a protein chain. This process takes place on ribosomes.

transposon (transposable, or movable genetic element). A relatively small DNA segment that has the ability to move from one chromosomal position to another.

trypsin. A proteolytic enzyme that is secreted by the pancreas.

tryptone. A pancreatic digest of casein used as a nitrogen source in culture media formulated for isolating and cultivating bacteria and fungi.

variant. A culture exhibiting a stable phenotypic change whether genetic or epigenetic in orgin.

vector. A carrier or transmission agent. In the context of recombinant DNA technology, a vector is the DNA molecule used to introduce foreign DNA into host cells. Recombinant DNA vectors include plasmids, bacteriophages, and other forms of DNA.

vegetative propagation. Reproduction of plants using a nonsexual process involving the culture of plant parts such as stem and leaf cuttings.

virion. A virus particle consisting of a core of nucleic acid and a protein capsule.

viscometer. An insturment for measuring fluid viscosity.

viscosity. The resistance of fluid to flow.

X-gal (5-Bromo-4-chloro-3-indoyl-ß-D-galactopyranoside). This chemical is a histochemical substrate for ß-galactosidase. A blue color results when attacked by ß-galactosidase. It has a formula of $C_{14}H_{15}BrC1NO_6$ and a molecular weight of 408.6.

xenobiotic. A novel synthetic compound that microorganisms normally do not encounter in the environment.

yeast. A unicellular fungus that does not give rise to mycelial growth.

zeatin. A plant cytokinin hormone and growth factor extracted from corn.

About The Ohio Academy of Science

The mission of The Ohio Academy of Science, a non-profit organization of those interested in science and technology, *is to empower curiosity, discovery and innovation by stimulating interest in the sciences, engineering and technology, promoting and supporting research, encouraging interaction among and between disciplines, improving science education, disseminating scientific knowledge, and recognizing and publicizing high achievement in attaining these objectives*. Through its Junior Academy, Senior Academy and Central Office, The Ohio Academy of Science provides support activities, conducts annual meetings and science days, and publishes, worldwide, a journal and newsletter that report developments in science, engineering technology, and education.

An alliance for science. A force for change. The common meeting ground of the scientific activities of the State. A federation of disciplines. We are these and more.

In contrast to many organizations, The Ohio Academy of Science embraces ALL the scientific disciplines. Nearly anyone interested in science will benefit from membership in Academy.

We promote cooperation between academic and industrial scientists; provide abundant youth science opportunities through the Junior Academy program; conduct meetings to present scholarly papers; publish, worldwide, The Ohio Journal of Science; provide advice to public policy makers; recognize excellence in education and research; and improve science education through work with boards of education, federal and state agencies, teachers, students, and administrators. We foster the public understanding of science by publications, through work with the media, through cooperation with science museums and science centers, and by maintaining a public information and referral center. We affect public policy in industrial development, education and the environment by representing you on boards and commissions, by serving on governing boards of state and national organizations, through our Industrial Council, by fostering public discussion of critical issues through meetings and symposia, and by providing advice to the Ohio General Assembly and State agencies. The Academy is strategically placed to advance science in Ohio.

As a private not-for-profit corporation we are unbridled in our ability to cooperate with a wide array of partners -- institutions of higher education, corporations, other professional organizations and governmental agencies.

Significant social change has occurred since our founding in 1891. But one thing remains constant: the need to maintain and improve the climate for curiosity, discovery and innovation. This climate has been aided remarkably by the persistent, enthusiastic pursuit of the Academy's objectives: to stimulate interest in the sciences, to promote research, to improve instruction in the sciences, to disseminate scientific knowledge, to encourage interaction among and between all disciplines, and to recognize high achievements in attaining these objectives.

Membership benefits

(1) subscription to The Ohio Journal of Science, (2) copies of the OAS NEWS, (3) Annual Meeting and Program Abstracts, (4) support of The Ohio Junior Academy of Science, (5) membership in the BMI Federal Credit Union, (6) tax deductible donations, (7) recognition, honors, and awards, (8) representation of your interests on boards and commissions, (9) use of a science information and referral center, (10) association with leading scientists and engineers, and (11) invitations to lectures, seminars, workshops and courses.

Goals

Encouraging and assuring the discovery, understanding, dissemination and practice of science, mathematics and engineering

1. The Ohio Academy of Science shall encourage and assure the discovery, understanding, dissemination, and practice of science, mathematics, engineering, and related disciplines in the public and private schools, the universities and colleges, the museums, the mines, farms, factories, and service establishments of Ohio. The Ohio Academy of Science shall represent the interests of all individuals, corporations, and institutions that have a vital interest in science, science education, and the application of science and engineering through technology.

Facilitating Research and Education

2. The objectives of the Academy shall include facilitating research to widen the knowledge base of mankind, and to enhance the welfare of all citizens through more efficient applications of science and new technologies to production. The objectives shall include facilitating science education at all academic levels.

Promoting Interdisciplinary Interaction

3. The Ohio Academy of Science shall promote and assure the interaction of scientists and engineers across disciplinary lines by means of publications, symposia, workshops, annual statewide meetings, regional meetings and other regional initiatives.

Providing Advice to State Government

4. The Ohio Academy of Science shall monitor the advances of science and technology and shall advise the State of Ohio through committees of the legislature and through the office of the Governor on changing science priorities, in order to address the changing needs of Ohio citizens.

Promoting Innovation and Adoption of New Technologies

5. The Ohio Academy of Science shall facilitate interaction between research and development personnel in industrial and business enterprises with the researchers and development personnel of university departments and other research facilities in order to induce innovation in the production sectors of the Ohio economy and to proactively encourage rapid adoption of new and better technologies.

Monitoring and Reporting of Funding Opportunities

6. The Ohio Academy of Science shall monitor federal and state research agendas and inform Ohio research groups of opportunities and research undertakings that will be funded at least partially by the federal and state government.

Widening Public Understanding and Appreciation of Science

7. The Ohio Academy of Science shall widen public understanding of science through existing communication channels, and shall endeavor to create in Ohio a value system that encourages creativity and innovation and public appreciation of scientists and engineers, science teachers and professors, and other technical people who apply science in their occupations. The Academy shall recognize and publicize high achievements in science, engineering and teaching. Moreover, the Academy shall encourage related career development programs and shall encourage graduate and post-graduate learning in the science disciplines.

Creating an Informed Citizenry

8. The Ohio Academy of Science recognizes that the applications of science, engineering and mathematics to the industries and arts of civilization are the mainspring of rising standards of living for the welfare of people everywhere; and that a citizenry informed in the purviews of science is the basis for appropriate public policy in a free society.

Reporting Developments in Science and Maintaining a Permanent Central Office

9. To accomplish the purposes of the mission The Ohio Academy of Science shall maintain a permanent central office, a junior academy; and a senior academy to provide support activities, including an annual meeting, science days, regional meetings, symposia and workshops; and shall publish The Ohio Journal of Science, and a newsletter, and special publications in order to report developments in science, engineering and technology.

Programs and Activities

The Ohio Senior Academy of Science:

Annual Meeting, Ohio Science Symposia, Ohio Flora Committee, Zoology Fund Student Paper Publication, Plant Science Student Paper Award, Geology Fund for Publication, Science Education University Educator Award

The Ohio Journal of Science:

Peer reviewed papers, Critical Perspectives in Pure and Applied Natural, Physical, and Social Sciences feature of OJS, OAS Newsletter, OJS Paper of the Year Award

The Ohio Junior Academy of Science:

1,300 Local Science Days, 16 District Science Days, State Science Day with more than $250,000 in scholarships and awards, Ohio's Space Scientists of Tomorrow Program, Student Research Grants, The John H. and Ruth W. Melvin Award for the American Junior Academy of Science Annual Meeting, Battelle Awards for Professional Development, Buckeye Science & Engineering Fair, Governor's Awards for Excellence in Youth Science Opportunities and for Student Research, Krecker School & Acker Outstanding Teacher Awards. Women in Science Programs including WISEMCO & B-WISER Institute and Summer Science Camp

The Ohio Business & Industrial Council:

Regional Industrial and Business Forums.

Special Projects:

Heartland Science and *Ohio's Weather Story* books, The Ohio Academy of Science Foundation, Science Education Projects and Publications.

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BIOTECHNOLOGY NOTES

About the Cover

Normal cell structures are illustrated on the front cover. In contrast, rapidly dividing, metastasizing cancer cells are depicted on the back cover. Normal cell transcription is emphasized on the front cover. Transcription factors melt the DNA double helix, while a DNA-dependent RNA-polymerase synthesizes RNA. These mRNA's encode structural proteins and maintenance enzymes. Cell division is held in check by tumor suppressor genes (oncogenes) such as p53 and Rb. Other illustrated features of the healthy cell are the nucleolus, mitochondria, cytoskeletin, the nuclear matrix, the porous nuclear membrane, the golgi, ribosomes, and the outer cellular membrane. The back cover shows metastasizing cancerous cells, which differ from the normal cell by their strikingly ruffled surfaces. Autocrine TGFα stimulation of the EGF receptor is shown adjacent to the neu protein, with both transversing a lipid bilayer cell membrane. Malignant cells are brightly illuminated by laser excited phycoerythrin antibody conjugates. Oncogene Science's extensive line of antibody products are represented by the antibody forms that float above the cells. Important genes and proteins such as p53, neu, rag, jun, fos, and cell cycle proteins can be identified and studied using these products. Our 400+ probes and complementary molecular biology products are depicted by the white star burst at the end of the DNA strand.

Cover art courtesy of Oncogene Science, Inc.

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